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PRINCIPAL INVESTIGATOR: John C. Reed, M.D.

CONTRACTING ORGANIZATION: The Burham Institute
La Jolla, California 92037

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LaJolla, CA 92037

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Two novel human genes were cloned that inhibit cell death and that therefore may contribute to breast cancer by preventing the normal cell turnover that is essential for keeping overall numbers of cells in the mammary gland within physiologically appropriate ranges. These genes suppress the pro-death activity of a protein called Bax. The Bax protein was shown previously to be present in normal mammary tissue but reduced or absent in ~one-third of breast cancers, in association with poor patient responses to chemotherapy and shorter overall survival. The cloning of these two new genes, termed BI-1 and BI-2 for Bax-inhibitors 1 and 2, may provide insights into how to restore the function of Bax in breast cancer which has reduced levels of this cell death promoting protein.

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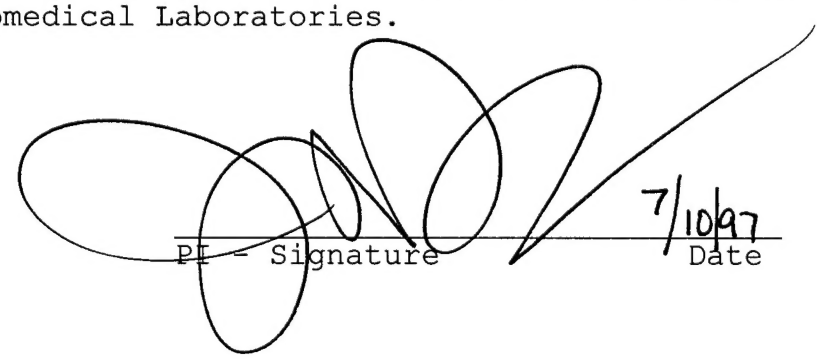

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1. INTRODUCTION

The Bcl-2 family of proteins regulates a distal step in an evolutionarily conserved pathway for programmed cell death and apoptosis, with some members of this family functioning as suppressors and others as promoters of cell death (reviewed in (1-3). Elements of this cell death pathway are genetically conserved from simple organisms such as the nematode *C. Elegans* and the fly *Drosphilia melanogaster* to mammals, including humans (4, 5). Bcl-2 is the first identified member of this family of homologous apoptosis-regulating proteins. Over-expression of Bcl-2 has been documented in many types of cancer, including adenocarcinomas of the breast (reviewed in Reed/J Clin Invest 1996). High levels of Bcl-2 contribute to tumorigenesis by blocking normal cell turnover due to programmed cell death, thus allowing for clonal expansion of tumor cells without concomitant elevations in cell division rates.

The actions of Bcl-2 are opposed in cells by certain other members of the Bcl-2 protein family which promote cell death rather than blocking it. Bax is the first identified pro-apoptotic member of the Bcl-2 family of proteins. This protein forms heterodimers with Bcl-2, abrogating the cytoprotective function of the Bcl-2 protein (6). Bax is expressed in normal mammary epithelium but is reduced or absent in ~35% of invasive breast cancers (7). In some types of epithelial cancers, mutations have been found within the *BAX* gene that inactivate it, implying that *BAX* is a tumor suppressor gene (8). Gene transfer-mediated restoraton of Bax protein expression in human breast cancer lines promotes cell death when triggered by a variety of initiators of apoptosis, such as growth factor withdrawal, ionizing radiation, and anti-Fas antibody (6, 9-11).

Bax appears to be important for tumor responses to chemotherapy and radiotherapy. For example, the Bax gene promoter contains typical p53 binding sites and is transcriptionally upregulated by p53 (12). Studies with Bax and p53 knock-out mice also suggest that Bax accounts for a substantial portion of p53's apoptosis inducing function in vivo (13). In this regard, reduced Bax expression has been correlated with poor responses to combination chemotherapy and shorter overall survival in women with metastatic breast cancer (7).

The loss of Bax expression in about one-third of breast cancers and the evidence that Bax is important for chemoresponses suggests a potential mechanism by which breast tumors may be refractory to therapeutic efforts. Thus, a need exists to better understand how the Bax protein functions and how one might attempt to restore Bax function in breast cancer cells.

1.2 Mechanisms of Bax protein function. The lack of aminoacid sequence homology between members of the Bcl-2 family and other proteins whose biochemical mechanism is known has hindered attempts to understand how Bcl-2, Bax, and their homologs regulate cell death. Both Bcl-2 and Bax, as well as most other members of the Bcl-2 family, contain a stretch of hydrophobic aminoacids near the C-termini that anchor the proteins in intracellular membranes. The Bcl-2 protein resides in the outer mitochondrial membrane, nuclear envelope, and endoplasmic reticulum (ER), whereas Bax appears to be restricted primarily to mitochondrial membranes (14-16). Immunoelectron microscopic studies suggest that Bcl-2 is concentrated at the junctional complexes in mitochondria and nuclear pore complexes in the nuclear envelope, raising the possibility that it controls some aspect of transport at these sites where the inner and outer membranes of these organelles come into contact and where various transport phenomena involving ions and proteins occur (14, 17).

Interestingly, the 3-dimensional structure of the anti-apoptotic Bcl-2 family protein Bcl-X_L has close structural similarity to the pore-forming domains of certain bacterial toxins, including diphtheria toxin and the colicins A and E1 (18). Moreover, we and others have obtained evidence from measurements of ion efflux from unilammelar liposomes and from single channel recordings in planar lipid bilayers that Bcl-2, Bcl-X_L, and Bax can form ion channels in membranes (19, 20) and S. Korsmeyer, personal communication). The diameter of the channels formed by these Bcl-2 family proteins and the molecules they are intended to transport in vivo however are unknown at present. In this regard, while diphtheria toxin's job is to transport a protein across biological membranes, namely the ADP-ribosylating subunit of the toxin, the colicins form non-specific ion-channels that kill bacteria by depolarizing them (21, 22). Thus, it is possible that Bcl-2 family proteins participate in the transport of either proteins or ions. Moreover, since both Bcl-2 and Bax can form channels, it has been suggested that they may transport proteins or ions in opposing directions, thereby accounting for the cytoprotective effects of Bcl-2 and the cytotoxic actions of Bax (19).

Among the biochemical events that occur after over-expressing Bax in cells are the induction of mitochondrial permeability transition pore (PTP) opening and activation of ICE/ced-3 family proteases (caspases) (23, 24). Mitochondrial PTP represents a very early event associated with apoptosis and may be a central event where a wide variety of upstream apoptosis initiating events converge into a final common pathway (reviewed in (25-27). As opposed to Bax which induces mitochondrial PTP, Bcl-2 prevents its induction. At least three potentially lethal events occur following the induction of mitochondrial PTP, with

its subsequent dissipation of the electrochemical gradient, swelling of the matrix and eventual rupture of the outer mitochondrial membrane: (1) reactive oxygen species (primarily superoxide) are generated due to interrupted electron chain transport; (2) Ca^{2+} sequestered in the matrix is dumped into the cytosol; and (3) apoptogenic proteins that are stored in the intermembrane space and which activate ICE/ced-3 proteases (caspases) are released (27-30).

Over-expression of Bcl-2 has been shown to block all three of these events associated with apoptosis or mitochondrial PTP(30-32), supporting arguments that regulation of mitochondrial PTP may represent a major function of Bcl-2. Interestingly, studies in which caspase activation was prevented by addition to cells of peptidyl inhibitors of these cysteine proteases indicate that over-expression of Bax nevertheless induces mitochondrial PTP and production of superoxides and also still induces cell death, but without the usual proteolytic events and without the oligonucleosomal DNA fragmentation that typically accompanies apoptotic cell death (23). Under these circumstances where proteases are blocked, Bax induces cytosolic vacuolarization and morphological changes more consistent with necrosis than apoptosis. Thus, by controlling mitochondrial PTP, Bcl-2 and Bax may be able to modulate both apoptosis and necrosis, with the former involving the obligatory actions of the caspases and the latter emphasizing oxygen free-radical production.

The Bcl-2 and Bax proteins can homodimerize and heterodimerize with each other, as well as with certain other members of the Bcl-2 protein family (6, 33). A variety of mutagenesis studies have suggested that some of these homo- and heterodimerization events may be important for the function of Bcl-2 and Bax, but overall have created a confusing picture. Mutations in Bax which prevent homodimerization ablate its cytotoxic function in many circumstances but not others (16, 34, 35). Similarly, mutations in Bax that prevent its binding to Bcl-X_L or Bcl-2 often but do not always abrogate the pro-apoptotic effects of this protein in cells (16, 36). Likewise, many but not all mutations in Bcl-2 or Bcl-X_L that prevent binding to Bax abrogate the survival-promoting effects of these anti-apoptotic proteins (37-40). The relevance to these various homo- and heterodimerization phenomenon to channel formation by Bcl-2 and Bax has yet to be addressed.

1.3. Bax exhibits cytotoxic function in yeast. Approximately 3 years ago, we discovered that Bax induces cell death when expressed in the budding yeast *S.cerevisiae* (16, 33, 39). Like in mammalian cells, the lethal phenotype conferred by Bax can be suppressed by anti-apoptotic Bcl-2 family proteins including Bcl-2 and Bcl-X_L, but not by mutant versions of these proteins which failed to heterodimerize with Bax. Based on EM analysis, the cell death that occurs in Bax-expressing yeast resembles that seen in mammalian cells when treated with pan-inhibitors of caspases, with massive cytosolic vacuolarization and only punctate chromatin condensation without the nuclear fragmentation and chromatin margination typical of apoptosis (16, 24). Consistent with this more necrotic morphology, we were unable to find evidence of caspase-like proteolytic activities in yeast (24). Experiments are underway to determine whether Bax induces mitochondrial PTP in yeast, but mutagenesis experiments in which the membrane-anchoring domain of Bax was deleted or swapped with mitochondria outer membrane-targeting domains from other proteins provided evidence that association with mitochondrial membranes is critical for Bax's cytotoxic activity in yeast (16).

Based on these observations, we proposed in the original proposal to develop genetic screens in yeast for identification of suppressors of Bax-mediated lethality. As described in the RESULTS section below, mammalian cDNA libraries under the control of a yeast promoter were introduced into *S. cerevisiae* which expressed mouse Bax under the control of a conditional Gal10 promoter and cDNAs that abrogated Bax-induced cytotoxicity were identified. This screening approach resulted in two human cDNAs that encode proteins which can interfere with the function of Bax in both yeast and mammalian cells.

It is likely therefore that the two proteins identified represent downstream participants or modifiers in the Bax-regulated pathway for cell death. As such, the identification of these novel genes provides a new starting point for understanding more about the biochemical and cellular functions of Bax which may eventually contribute to improved treatments for breast cancer.

2. RESULTS

3.1 Cloning of cDNAs encoding BI-1 and BI-2.

Previous studies in our laboratory and other's have shown that expression of the pro-apoptotic gene Bax induces yeast cell death, both in *S. cerevisial* budding yeast (16, 33, 39, 41) and in *S. pombe* fisson yeast (24). The library screening described here was aimed at searching for human cDNAs that can rescue yeast cells from Bax-induced death. For this study, *S. cerevisiae* yeast strain QX95001 was constructed by transforming the Bax-bearing plasmid YEp51-Bax into yeast strain BF264-*15Dau (MATα ade1 his2 leu2-3,112 trp1-1a ura3*; (42). YEp51-Bax (16) is a *URA3*-marked yeast high-copy plasmid containing a full-length mouse Bax cDNA under the control of the galactose-

inducible yeast *GAL10* promoter. QX95001 cells were verified to express Bax and die upon being shifted from glucose to galactose-containing medium (not shown).

A human HepG2 cDNA library, under the control of the yeast *GAP1* promoter and therefore expressible in yeast cells, (43) was used for library screening. Briefly, QX95001 cells were grown to mid-log phase (~$2x10^7$ cells/ml) in standard YPD liquid medium (44) and ~100 µg of the $HepG_2$ cDNA library plasmids DNA were introduced by a Lithium Acetate transformation procedure (44). Bax-resistant transformants were directly selected on galactose-containing solid medium. An aliquot of transformation mixture was also spread on glucose-containing medium to determine transformation efficiency. Initially 75 Bax-resistant colonies were obtained from a screen of ~10^6 transformants (assayed at five-days after transformation). These yeast colonies were patched onto galactose medium as a secondary screen to verify their ability to overcome Bax-induced cytotoxicity, and 17 proved to be Bax-resistant.

Bax-resistant colonies could result either from the presence of a human cDNA in the transformed yeast cells, or from a yeast gene mutation that suppresses Bax function in these cells. To distinguish between these possibilities, a "concommitant -loss" assay was applied. The rational is that if the Bax-resistance phenotype was caused by a library cDNA , then the loss of the library plasmid (indicated by the concommitant loss of the plasmid marker, *URA3*) would result in loss of the Bax-resistance phenotype. In contrast, if Bax-resistance was caused by an endogenous yeast gene mutation, then the loss of the library plasmid should not have any impact on the Bax-resistance phenotype. This "Con-loss" test was carried out for the above 17 positive clones, with 4 of them (clones 6, 8, 25, and 32) exhibiting the expected phenotype. The ability of remaining 13 clones to survive Bax killing is most likely due to mutations in the yeast strain and was not pursued further in this study.

Plasmid DNA was recovered from the 4 "Con-loss" positive clones using the "Glassbeads" method (44) and re-introduced into strain QX95001 which contains the galactose inducible YEp51-Bax plasmid to again verify that these plasmids were indeed responsible for suppressing Bax-induced yeast cell death (Figure 1). Comparisons were made in Figure 1 between yeast retransformed with the BI-1 (pQX36-1) and BI-2 (pQX36-2) plasmids versus the original yeast clones obtained during the cDNA library screen (clones 6 and 8).

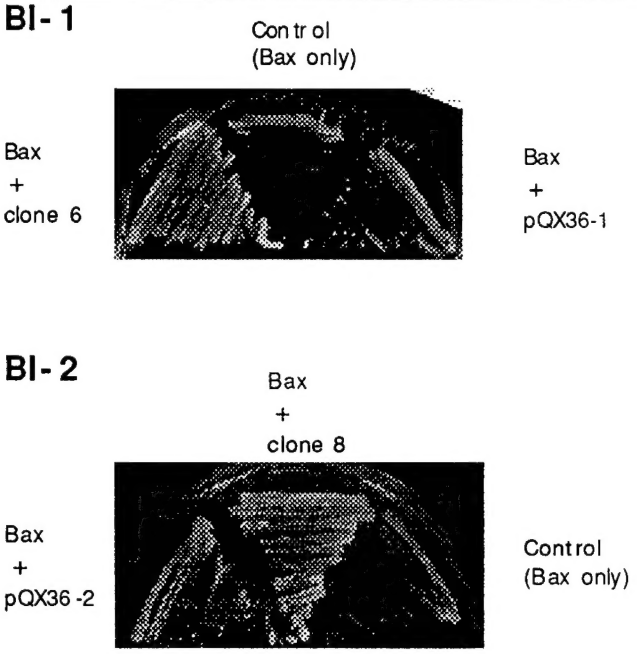


Figure 1

Nucleotide sequence analysis of these cDNAs revealed that three of them encoded overlapping fragments of the same cDNA. These 3 cDNAs all contained the complete ORF for a 237 amino acid protein: named BI-1 (for Bax-inhibitor 1). The fourth clone was unique and named BI-2 for Bax-inhibitor-2. The BI-2 cDNA encodes a predicted protein of 450 amino acids (Figure 2).

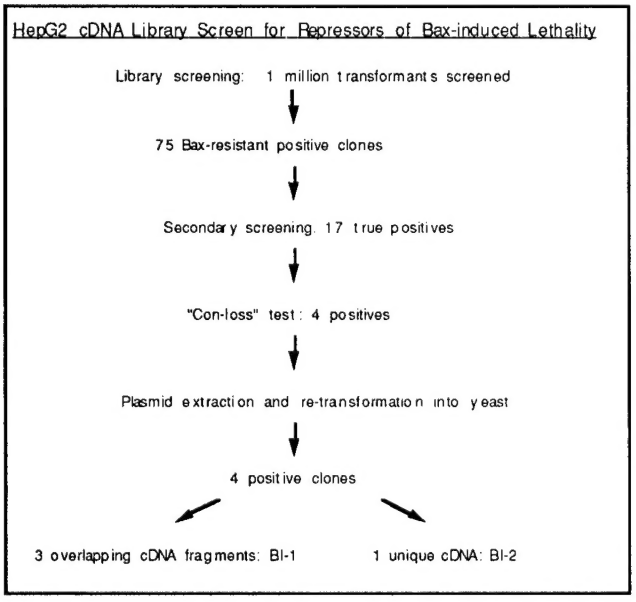
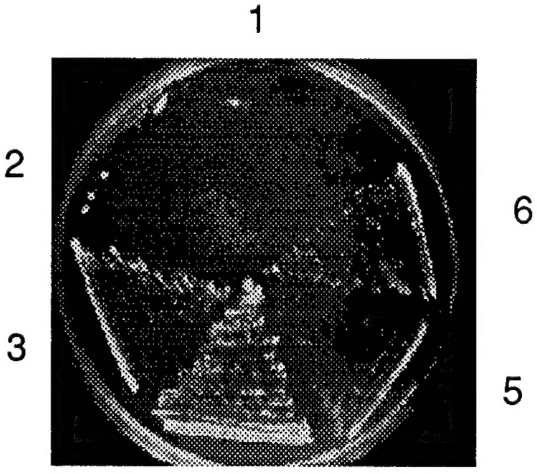


Figure 2

3.2. BI-1 and BI-2 do not suppress production of Bax protein in yeast.

One potential mechanisms by which BI-1 and BI-2 could suppress Bax-induced lethality in yeast is by inhibiting expression of the *Gal10* promoter which drives galactose-inducible expression of Bax in QX95001 strain cells. To exclude this trivial explanation, therefore, the BI-1-encoding plasmid pQX36-1 (1μg) and the BI-2 encoding plasmid pQX36-2 (1μg) were co-transformed into EGY48 strain yeast with pEG202-Bax (1μg), a yeast plasmid that contains the mouse *bax* cDNA under the control of the strong constitutive *ADH* promoter (33). As shown in Figure 3, the BI-1- and the BI-2-encoding plasmids suppressed Bax-induced killing, despite the fact that Bax was expressed from a different promoter that is independent of galactose, allowing the cells to grow when streaked on appropriate solid medium (i.e., minimal drop-out medium lacking uracil and histidine to select for the pQX and pEG202 plasmids, respectively).



1, 2: ADH1-bax + Vector
3, 4: ADH1-bax + pQX36-2 (BI-2)
5,6: ADH1-bax + pQX36-1 (BI-1)

Figure 3

To directly confirm that BI-1 and BI-2 do not suppress production of Bax protein in yeast, Western blot analysis was performed using extracts that were prepared from QX95001 strain yeast (which express Bax under the control of the galactose-inducible Gal10 promoter) after 20 hrs of growth in synthetic liquid medium containing either glucose (also known as dextrose) (D) or galactose (G). Lysates were normalized for total protein content (25 ug per lane), and subjected to SDS-PAGE/immunoblot assay using anti-mouse Bax antiserum (45). As shown in Figure 4, similar levels of Bax protein were present in yeast when grown in galactose-containing medium, regardless of whether the cells contained the BI-1- or BI-2-expressing plasmids pQX36-1 or pQX36-2, respectively.

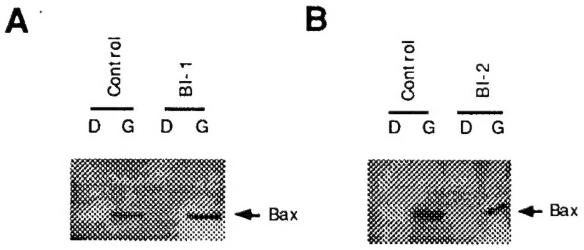


Figure 4

3.3. DNA sequence analysis of BI-1 cDNAs.

The three BI-1 encoding clones (clones 6, 25, 32) (longest 2633 bp) all contained a single long open reading frame (ORF) for a predicted protein of 237 aminoacids. This ORF is proceeded by an upstream inframe stop codon, located at -57 bp within the predicted 5' untranslated region. Though no motifs or similarities with other proteins were found that might suggest a biochemical function for BI-1, a Kyte-Doolittle plot of protein hydrophobicity revealed six predicted membrane spanning domains (Figure 5). The only other known apoptosis-regulating proteins with a similar topology are the presenilin-1 and -2 proteins, which have been implicated in Alzheimer's disease due to their mutations in familial Alzheimer's disease (46-48). Like BI-1, PS-1 and PS-2 are predicted to have a serpentine topology and contain 6 to 9 membrane spanning domains. However, BI-1 does not share significant primary aminoacid sequence homology with PS-1 or PS-2.

BI-1

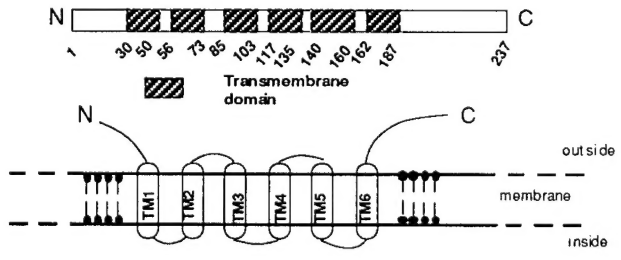


Figure 5

3.4 BI-1 protects mammalian cells from Bax-induced apoptosis.

Previously, we have shown that transient transfection of Bax-encoding plasmids into the human embryonic kidney cell line 293 induces apoptosis and activation of ICE-family proteases (caspases) (16, 24). Using this assay, we explored the function of BI-1 in human cells. The plasmid pcDNA-3-Bax was co-transfected with equivalent amounts of pcDNA-3 parental vector (used as a negative control), pcDNA3-BI-1 or pcDNA-3-Bcl-2 (used here as a positive control for suppression of Bax-induced apoptosis). As shown in Figure 6, Bax co-transfected with the negative control pcDNA-3 parental plasmid caused cell death in ~35% of the cells, based on trypan blue dye exclusion. In contrast, only 5% of cells were dead when Bax was not transfected (pcDNA-3 plasmid only ["vector"]). Co-transfection of the BI-1 expression plasmid with Bax reduced cell death by over half. The extent to which BI-1 abrogated Bax-induced cell death was similar to that seen when a Bcl-2-encoding plasmid was co-transfected with Bax (Figure 6).

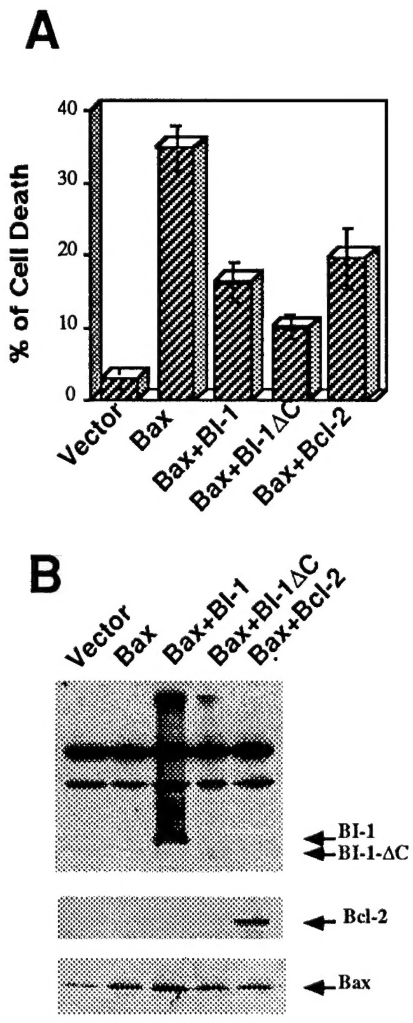


Figure 6

Western blotting confirmed production of the BI-1 protein and demonstrated that BI-1 does not interfere with Bax expression (Figure 6B). Interestingly, when a BI-1 mutant was expressed which is lacking the C-terminal cytosolic domain, suppression of Bax-induced cell death was enhanced even though this mutant protein was expressed only at very low levels (Figure 6B). Thus, this domain may represent a negative regulatory region that impairs BI-1 function. Co-transfections which included a β-galactosidase-encoding plasmid confirmed approximately equal efficiencies of transfection in all cases, with ~80% of the cells displaying β-galactosidase activity (not shown). Similar results were obtained when apoptotic cells were enumerated based on fluorescence microscopy using the DNA-binding fluorochrome DAPI (data not presented).

3.5 BI-1 inhibits apoptosis induced by growth factor deprivation.

Apoptosis induced by withdrawal of Interleukin-3 (IL-3) from the factor-dependent hemopoietic cell FL5.12 has been used extensively as a model for studying Bcl-2 family proteins. Gene transfer-mediated over-expression of Bcl-2 inhibits IL-3 withdrawal-induced cell death, whereas Bax accelerates it. To further explore the effects of BI-1 on regulation of apoptosis, FL5.12 cells were stably transfected with pcDNA-3-BI-1-HA and several clones were identified by immunoblotting (not shown) which expressed relatively high levels of the HA-tagged BI-1 protein: clones 2, 4, 5, and 8 (Figure 7). When deprived of IL-3, control cells that had been transfected with pcDNA3 began to die, with approximately half of the cells failing to exclude trypan blue dye within 24 hrs. In contrast, BI-1-expressing clones exhibited prolonged survival under the same conditions. The enhanced survival imparted by BI-1 overexpression however was not as great as that seen when Bcl-2 was over-expressed in FL5.12 cells. As an additional control, a clone of FL5.12 cells over-expressing Bax was also tested, demonstrating accelerated cell death in the absence of IL-3, as expected (Figure 7). Though preliminary, these data further support the hypothesis that BI-1 is a suppressor of apoptosis in mammalian cells.

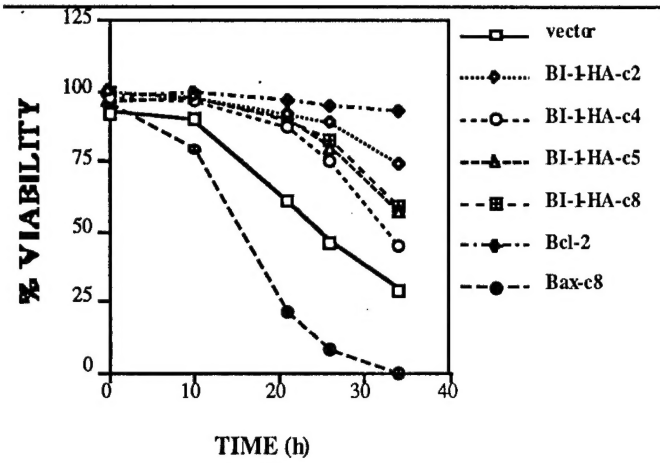


Figure 7

3.6 DNA sequences analysis of BI-2 cDNAs.

The BI-2 cDNA (2565 bp) is novel, sharing homology only with some short unidentified fragments in the EST database. The ORF of this cDNA encodes a predicted protein of 450 aminoacids and is proceeded by an upstream inframe stop codon at position -6 bp in the predicted 5'-untranslated region. This protein contains a RING domain near its N-terminus and has two predicted membrane-spanning domains, but otherwise lacks any motifs or similarity to other proteins (Figure 8).

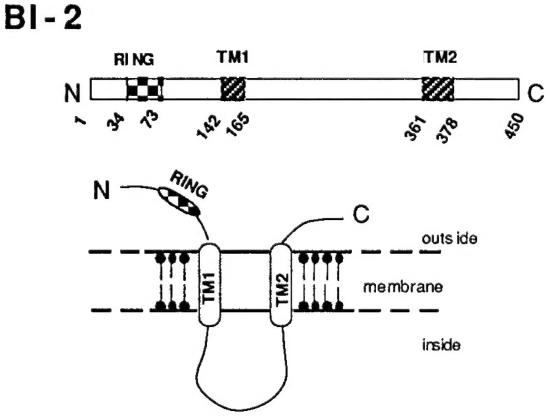


Figure 8

The presence of the predicted ORF in the BI-2 cDNA was confirmed by in vitro translation experiments. The BI-1 and BI-2 cDNAs were subcloned into pcDNA and the resulting plasmid DNAs (1 ug) were added to Promega TNT reticulocyte lysates containing T7 RNA polymerase and ^{35}S-L-methionine (~1000 uCi/mmole). The resulting proteins were analyzed by SDS-PAGE/autoradiography. Radiolabeled protein bands were observed which were consistent with the predicted masses of the BI-1 (~26.4 kDa) and BI-2 (~52.8 kDa) proteins (Figure 9). HA-tagged versions of these proteins were also prepared in the mammalian expression plasmid pcDNA-3. In vitro translation from the T7 RNA polymerase promoter resulted in production of the expected size proteins (have 3 copies of HA tag appended to N-terminus).

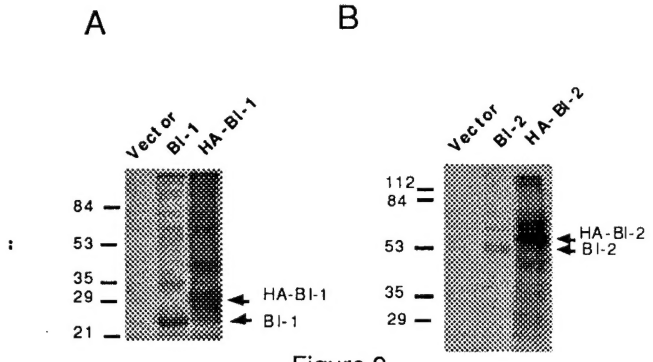


Figure 9

3.7 BI-2 inhibits Bax induced death in 293 cells.

To preliminarily explore the function of BI-2 in mammalian cells, the same Bax-transient transfection assay involving 293T cells was used as described above. As shown in Figure 10, co-transfection of BI-2 with Bax reduced Bax-induced cell death by approximately half. In contrast, a truncation mutant of BI-2 lacking the RING domain was inactive in this assay. Western blotting confirmed production of the full-length and ΔRING HA-tagged BI-2 proteins, and demonstrated that BI-2 does not interfere with expression of Bax (not shown). Though more work needs to be done, these preliminary observations suggest that BI-1 also inhibits Bax-induced cell death in mammalian cells.

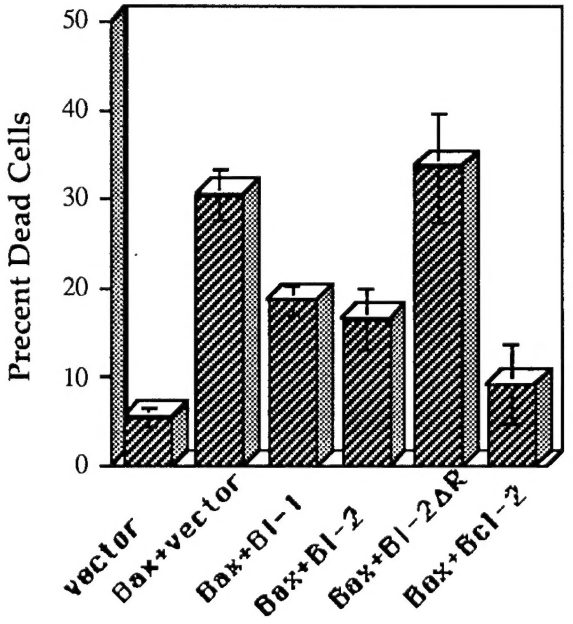


Figure 10

3.8 Subcellular location of BI-1 and BI-2 explored by Green Fluorescence Protein (GFP) tagging. Both BI-1 and BI-2 are predicted to be integral membranes proteins. To preliminarily explore the intracellular locations of BI-1 and BI-2, these proteins were expressed in several different cell lines (293, COS, GM701) as GFP fusions, using the expression plasmids pEGFP-N2 and pEGFP-C2 (Clontech, Inc.), respectively. These vectors encode a double-mutant of GFP which has brighter fluorescent properties than the wild-type GFP. The GFP was appended to the C-terminus of BI-1, whereas the GFP was fused to the N-terminus of BI-2. In all 3 cell lines, confocal fluorescence microscopy demonstrated that BI-1 almost exclusively associated with intracellular membranes in a pattern typical of the endoplasmic reticulum (ER) and its continuity with the nuclear envelope (not shown). In some cells, BI-1 was also associated with organelles resembling mitochondria, based on two-color analysis with mitochondria-specific dyes (not shown). In contrast, BI-2 displaced both a reticular cytosolic ER and nuclear envelope pattern, as well as a punctate cytosolic fluorescence in association with what appeared to be cytosolic organelles in all three cell lines. Again, these cytosolic organelles appeared to be mitochondria, based on two color analysis using a mitochondria-specific dye, MitoTracker (Molecular Probes, Inc.). Thus, BI-2 displayed an intracellular localization very similar to that previously reported for Bcl-2 and Bcl-X_L (14, 49), whereas BI-1 was distributed in a pattern more similar to the Bcl-2 homologs Mcl-1 and Bak (50); Robin Brown personal communication), and the Alzheimer's-associated proteins PS-1 and PS-2 (51).

3.9 Investigations of BI-1 location by subcellular fractionation studies.
To further explore the intracellular locations of the BI-1 protein, crude subcellular fractionation studies were performed using either control 293T cells or BI-1-HA-expressing cells, resulting in nuclear, cytosolic, light membrane, and heavy membrane fractions (52, 53). Immunoblot analysis of these fractions demonstrated the presence of BI-1-HA protein predominantly in the nuclear (N) and heavy membrane (HM) fractions, with only small amounts of BI-1 in the light membrane fraction and none in the cytosol. The smearing of BI-1 seen in the gel presumably can be attributed to its extremely hydrophobic nature. Reprobing the same blot confirmed the presence of the mitochondrial protein F_1 β ATPase, the nuclear protein PARP, and the cytosolic protein CPP32 exclusively in the expected fractions, i.e.: HM, N, and C, respectively (Figure 11). Bcl-2 was found predominantly in the HM fraction where mitochondria reside, with some protein also associated with the nuclear fraction as a result of Bcl-2 integration into the nuclear envelope (53). Bax was also present primarily in the mitochondria-containing HM fraction, with some also in the LM. Note, however, that a substantial portion of Bax appeared in the cytosolic fraction, consistent with a recent report that demonstrated a cytosolic pool of Bax which can become mitochondria associated during apoptosis (54). Importantly, BI-1 did not alter the ratio of Bax protein associated with the cytosol versus membrane fractions, thus excluding this as a potential mechanism for BI-1-mediated suppression of Bax killing.

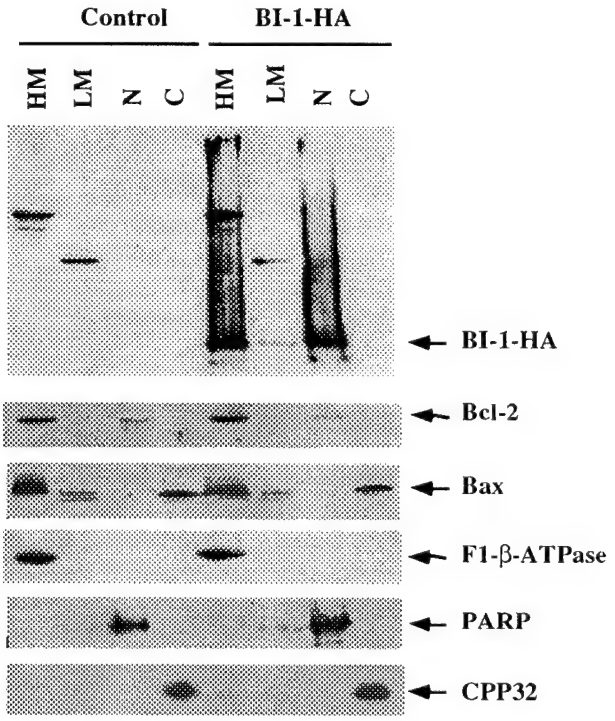


Figure 11

3.10 BI-1 is an integral membrane protein.
Triton X114 partitioning studies were performed to confirm the expected integral membrane nature of BI-1. Using lysates from control and BI-1-HA-expressing 293T cells, BI-1 was observed to partition almost exclusively into Triton X114 (Figure 12).

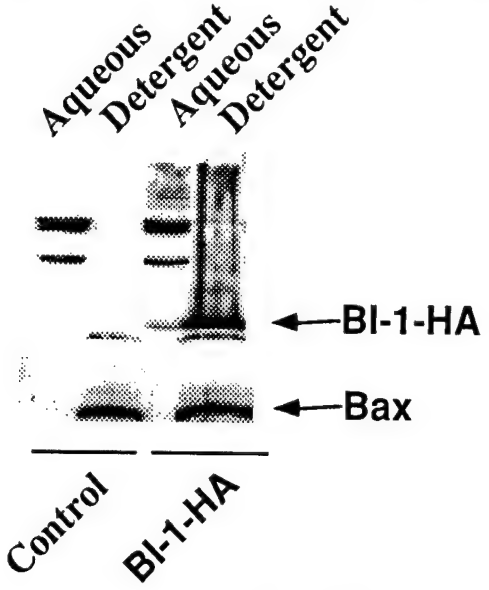
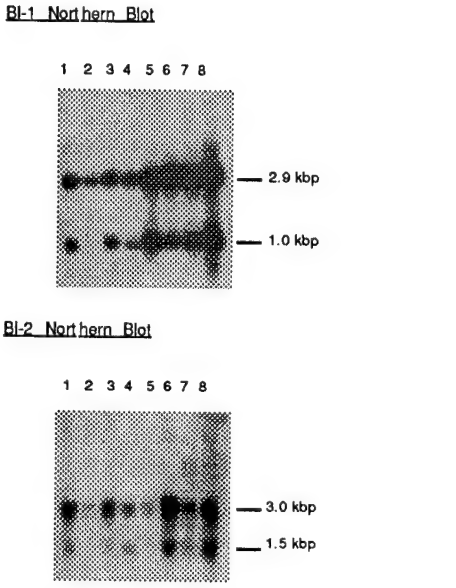


Figure 12

3.11 BI-1 and BI-2 are widely expressed. Northern blot analysis was used to explore the tissue-distribution of BI-1 and BI-2 expression. Both BI-1 and BI-2 were expressed in most tissues, including the brain (Figure 13). Interestingly, two different size transcripts were detected for both BI-1 and BI-2, with major mRNAs of 2.9 kbp and 1.0 kbp seen for BI-1 and mRNAs of 3.0 kbp and 1.5 kbp for BI-2. Moreover, only the 2.9 kbp BI-1 and the 3.0 kbp BI-2 transcripts appeared to be expressed in brain. It remains to be determined whether these different size transcripts arise from alternative splicing mechanisms and whether they encode different proteins.



Lanes 1-8 contain, in order, poly A+ RNA from (1) human heart, (2) brain, (3) placenta, (4) lung, (5) liver, (6) skeletal musde, (7) kidney, and (8) pancreas.

Figure 13

3.12. Bax and Bak confer a lethal phenotype in fission yeast, *S. pombe*. Approximately 3 years ago, we discovered that Bax induces cell death when expressed in the budding yeast *S.cerevisiae* (16, 33, 39). As part of this grant proposal, we also investigated whether Bax and its close relative Bak could kill the fission yeast *S. pombe* (24). Since fission yeast replicate by a mechanism more similar to mammalian cells, we wanted to be poised to take advantage of this organism for genetic screening if budding yeast provided to be unsuitable.

These studies demonstrated that the pro-apoptotic protein Bax and Bak are potent killers of fission yeast, and that the anti-apoptotic proteins Bcl- 2 and Bcl-XL can rescue *S. pombe* from Bax and Bak. It was also shown that, unlike mammalian cells, Bax and Bak induced cell death in fission yeast does not involve caspases and is not inhibitable by the caspase-inhibitor p35. These studies thus established that the cytotoxic functions of Bax and Bak are evolutionarily conserved in both budding and fission yeast, and highlighted that the cell death in these organisms is not coupled to activation of cell death proteases but rather presumably derives from the intrinsic activity of these proteins as putative ion channels which reside in mitochondrial membranes.

3. CONCLUSIONS

Breast cancers arise not only because of defects in the cell division cycle, but also because of a failure of normal cell turnover due to blocked rogrammed cell death. The Bax protein is a potent inducer of cell death, whose expression becomes lost or markely reduced in chemoresistant breast cancers. The need exists therefore to understand more about Bax and the mechanism by which it promotes apoptosis.

The goals of the originally proposed IDEA grant have been met. Two novel genes that inhibit the induction of apoptosis by Bax were cloned, BI-1 and BI-2. The BI-1 and BI-2 proteins provide new starting points for understanding the mechanisms that regulate cell life and death in breast cancers and for delineating the functions of the Bax protein. Manuscripts describing the cloning and characterization of the BI-1 and BI-2 proteins are in preparation.

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Bax- and Bak-induced Cell Death in the Fission Yeast *Schizosaccharomyces pombe*

Juliane M. Jürgensmeier,* Stanislaw Krajewski,* Robert C. Armstrong,† Gary M. Wilson,† Tilman Oltersdorf,† Lawrence C. Fritz,† John C. Reed,*‡ and Sabine Ottilie†‡§

*The Burnham Institute, Apoptosis Research Program, La Jolla, California 92037; and †IDUN Pharmaceuticals, Inc., La Jolla, California 92037

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The effects of the expression of the human Bcl-2 family proteins Bax, Bak, Bcl-2, and Bcl-X_L were examined in the fission yeast *Schizosaccharomyces pombe* and compared with Bax-induced cell death in mammalian cells. Expression of the proapoptotic proteins Bax and Bak conferred a lethal phenotype in this yeast, which was strongly suppressed by coexpression of the anti-apoptotic protein Bcl-X_L. Bcl-2 also partially abrogated Bax-mediated cytotoxicity in *S. pombe*, whereas a mutant of Bcl-2 (Gly145Ala) that fails to heterodimerize with Bax or block apoptosis in mammalian cells was inactive. However, other features distinguished Bax- and Bak-induced death in *S. pombe* from animal cell apoptosis. Electron microscopic analysis of *S. pombe* cells dying in response to Bax or Bak expression demonstrated massive cytosolic vacuolization and multifocal nuclear chromatin condensation, thus distinguishing this form of cell death from the classical morphological features of apoptosis seen in animal cells. Unlike Bax-induced apoptosis in 293 cells that led to the induction of interleukin-1β-converting enzyme (ICE)/CED-3-like protease activity, Bax- and Bak-induced cell death in *S. pombe* was accompanied neither by internucleosomal DNA fragmentation nor by activation of proteases with specificities similar to the ICE/CED-3 family. In addition, the baculovirus protease inhibitor p35, which is a potent inhibitor of ICE/CED-3 family proteases and a blocker of apoptosis in animal cells, failed to prevent cell death induction by Bax or Bak in fission yeast, whereas p35 inhibited Bax-induced cell death in mammalian cells. Taken together, these findings suggest that Bcl-2 family proteins may retain an evolutionarily conserved ability to regulate cell survival and death but also indicate differences in the downstream events that are activated by overexpression of Bax or Bak in divergent cell types.

INTRODUCTION

Programmed cell death (PCD) plays important roles in tissue homeostasis and developmental elimination of redundant or excess cells in essentially all multicellular organisms (reviewed in Ellis *et al.*, 1991; Ucker, 1991; Williams, 1991; Korsmeyer, 1992; Reed, 1994). In many cases, PCD involves a characteristic set of morphological events, known as apoptosis, which include plasma membrane blebbing, cell shrinkage, chromatin condensation, and nuclear fragmentation, followed ultimately by the budding off of cellular fragments which are cleared by phagocytosis (Wyllie *et al.*, 1980). Apoptosis is also often accompanied by DNA fragmentation, resulting from the activation of endonucleases which initially cleave the genomic DNA at its most assessable locations, between the nucleosomes, thus generating an oligonucleosomal degradation pattern which appears as a "ladder" of DNA bands that can be detected by conventional agarose gel electrophoresis (Wyllie *et al.*, 1984). Many of the morpholog-

‡ Reprint requests may be addressed to either Dr. Ottilie or Dr. Reed. IDUN Pharmaceuticals, Inc., 11085 N. Torrey Pines Rd., La Jolla, CA 92037.
§ Corresponding author.

ical features of apoptotic cells may also be the result of specific proteolytic cleavage events mediated at least in part by the interleukin-1β-converting enzyme (ICE)/CED-3 family of cysteine proteases (reviewed in Martin and Green, 1995). These proteases have specificity for aspartic acid in the P1 position (aspases) and mediate, directly or indirectly, the cleavage of a number of cellular proteins during apoptosis, including those associated with the nuclear matrix (PARP, topoisomerase I), nuclear envelope (lamins, SREBPs), and cytoskeleton (actin, gas-2, fodrin; Ucker *et al.*, 1994; Brancolini *et al.*, 1995; Martin *et al.*, 1995; Tewari *et al.*, 1995; Kayalar *et al.*, 1996; Wang *et al.*, 1996).

One of the distinctive features of PCD and apoptosis is the cell autonomous nature with which these forms of cell death occur, with the dying cells serving as active participants in their own demise (i.e., committing suicide; Ucker, 1991). Cell suicide behaviors have been observed even in some unicellular organisms, raising speculations that the cell death mechanisms currently recognized as apoptosis and PCD in multicellular organisms may have very primitive evolutionary origins (reviewed in Shub, 1994; Yarmolinsky, 1995; Ameisen, 1996). For example, some strains of *Escherichia coli* will activate the expression of genes that trigger cell death when infected with bacteriophages, presumably as a mechanism for limiting viral replication and thus ensuring the survival of the clonal descendants of an infected bacterium. Moreover, many other examples of cell death exist in unicellular bacterial species that could reasonably be classified as suicide or programmed cell death in that the cell is an active participant in its own demise and the death of individual cells affords survival value to other cells that derive from common antecedents, including the cell deaths associated with 1) restriction modification systems involving restriction endonucleases and counteracting methylases; 2) cell suicide following DNA damage induced by irradiation or chemicals in bacteria carrying colicin toxin genes; 3) the death of "mother cells" during sporulation of bacilli species; and 4) the death and subsequent cannibalization of cells during fruiting body formation by myxobacteria (reviewed in Shub, 1994; Yarmolinsky, 1995). In addition, cell death occurring with the classical features of apoptosis has also been reported in some eukaryotic unicellular organisms, such as in *Trypanosoma* species which have been shown to undergo apoptotic cell death in response to high cell densities or insufficient nutrients (Ameisen *et al.*, 1995; Welburn *et al.*, 1996).

Several genes that regulate programmed cell death and apoptosis have been identified in both vertebrate and invertebrate metazoan species, including nematodes, flies, mice, and humans (reviewed in Vaux and Strasser, 1996). The finding of significant sequence homology among some of these cell death-regulatory genes and their encoded proteins has suggested the existence of an evolutionarily conserved pathway that controls cell survival and death (reviewed in Ellis, 1991; Vaux and Strasser, 1996). This notion is further supported by the discovery of structurally and functionally related genes in several viruses that infect mammalian or insect species (Weiss *et al.*, 1987; Rao *et al.*, 1992; Henderson *et al.*, 1993; Clem and Miller, 1994). Furthermore, the discovery that some PCD-regulatory genes have homologues in both plants and animals (such as the anti-apoptotic gene *dad-1*) suggests that some portions of the cell death machinery may have evolved originally in unicellular organisms, before the divergence of the plant and animal kingdoms (Apte *et al.*, 1995).

Among the more prominent regulators of apoptosis are members of the Bcl-2 family of proteins. These proteins appear to regulate a distal step in what may represent a final common pathway for programmed cell death and apoptosis. Some of the members of this protein family function as suppressors of apoptosis, whereas others act as promoters of cell death (reviewed in Vaux, 1993; Reed, 1994; Nuñez and Clarke, 1994; Oltvai and Korsmeyer, 1994). Interestingly, although best known for its ability to regulate apoptotic cell death, the anti-apoptotic protein Bcl-2 has also been reported to be capable of suppressing necrotic cell death under some circumstances (Kane *et al.*, 1993, 1995; Subramanian *et al.*, 1995; Shimizu *et al.*, 1996), suggesting that some overlap exists in the cell death mechanisms which lead to apoptosis and necrosis and implying that Bcl-2 family proteins can potentially influence both of these types of cellular demise. At present, the mechanism by which Bcl-2 family proteins control cell life and death remains enigmatic, principally because the predicted amino acid sequences of these proteins share no significant similarity with other proteins that have a known biochemical activity. The three-dimensional structure of the Bcl-X_L protein, as determined by nuclear magnetic resonance (NMR) and x-ray crystallographic studies, however, is highly reminiscent of some types of pore-forming bacterial toxins, particularly the B-subunit of diphtheria toxin and *E. coli* colicin-A (Muchmore *et al.*, 1996).

Recent studies indicate that overexpression of Bcl-2 or Bcl-X_L, anti-apoptotic members of this family of proteins, can prevent the proteolytic processing and activation of ICE/CED-3 family proteases such as CPP32 during induction of apoptosis in mammalian cells (Armstrong *et al.*, 1996; Boulakia *et al.*, 1996; Chinnaiyan *et al.*, 1996; Messmer *et al.*, 1996). Furthermore, genetic analysis of the free-living nematode, *Caenorhabditis elegans*, has revealed a requirement for *ced-9*, the worm homologue of *bcl-2*, for suppression of programmed cell deaths mediated via a CED-3 protease-dependent pathway (Yuan *et al.*, 1993; Hengartner and Horvitz, 1994). Bcl-2 family proteins therefore appear to act at a step at or upstream of the ICE/CED-3 family

proteases. Whatever the biochemical process by which Bcl-2 family proteins modulate cell survival and death, elements of this mechanism appear to be well conserved throughout evolution based on the ability of the human Bcl-2 protein to 1) protect insect cells from virus-induced apoptosis (Alnemri *et al.*, 1992); 2) rescue superoxide dismutase- (*sod*) deficient strains of budding yeast (*Saccharomyces cerevisiae*) from cell death induced by growth under aerobic conditions (Kane *et al.*, 1993); and 3) partially substitute for *ced-9*, the nematode homologue of *bcl-2*, in suppressing cell death during development in *C. elegans* (Hockenbery *et al.*, 1990).

The function of Bcl-2 family proteins appears to be regulated, at least in part, by their interactions with each other through a complex network of homo- and heterodimers (Oltvai *et al.*, 1993; Sato *et al.*, 1994; Yin *et al.*, 1994; Bodrug *et al.*, 1995; Hanada *et al.*, 1995; Sedlak *et al.*, 1995). The anti-apoptotic proteins Bcl-2 and Bcl-X_L, for example, bind to the proapoptotic protein Bax and suppress cell death (Oltvai *et al.*, 1993; Sato *et al.*, 1994; Sedlak *et al.*, 1995). Mutant versions of Bcl-2 and Bcl-X_L have been described that fail to heterodimerize with Bax and also lack anti-apoptotic function (Oltvai *et al.*, 1993; Sedlak *et al.*, 1995). Similarly, the wild-type Bak protein, a proapoptotic member of the Bcl-2 protein family, binds to Bcl-X_L and promotes cell death, whereas mutant versions of Bak that are incapable of heterodimerizing with Bcl-X_L are deficient in promoting apoptosis (Chittenden *et al.*, 1995a). In addition, loss-of-function mutants of the Bcl-2 and Bax proteins have been described which have impaired ability to homodimerize (Hanada *et al.*, 1995; Zha *et al.*, 1996a).

The goal of delineating the molecular mechanisms of Bcl-2 family proteins would be greatly facilitated by being able to assess the function of these apoptosis-regulating proteins in more simple eukaryotes that are easily manipulated in terms of genetic analysis. Recently, it has been shown that the proapoptotic protein Bax confers a lethal phenotype when expressed in the budding yeast *S. cerevisiae* (Sato *et al.*, 1994, 1995; Greenhalf *et al.*, 1996) and is primarily targeted to the mitochondrial membranes (Zha *et al.*, 1996b). Analogous to studies performed in mammalian cells, anti-apoptotic members of the Bcl-2 protein family, including Bcl-2, Bcl-X_L, and Mcl-1, suppress the lethal function of Bax in yeast, whereas the proapoptotic protein Bcl-X_S and various deletion mutants of Bcl-2 which are nonfunctional in mammalian cells do not (Sato *et al.*, 1994; Bodrug *et al.*, 1995; Hanada *et al.*, 1995; Greenhalf *et al.*, 1996). Interestingly, even some mutants of Bcl-2 which retain the ability to bind Bax but which are deficient in anti-apoptotic activity in mammalian cells are likewise unable to suppress Bax-mediated lethality in budding yeast (Hanada *et al.*, 1995), implying that the mere binding of anti-apoptotic Bcl-2 family proteins to Bax can be insufficient to abrogate its function in yeast.

These parallels between the effects of wild-type and mutant Bcl-2 family proteins in budding yeast on cell death suggest that some aspects of the functions of these proteins may be translatable into less complex organisms. Although few details are known concerning the mechanisms by which Bax kills and Bcl-2 protects in budding yeast, preliminary explorations of this topic suggest that it does not involve apoptosis (Nuñez and Clarke, 1994). Since aspects of the cell division mechanisms utilized by fission yeast more closely resemble what occurs in mammalian cells (Alfa *et al.*, 1993), we explored the function of the Bcl-2 family proteins Bax, Bak, Bcl-2, and Bcl-X_L in *S. pombe*. In addition, we extended previous studies of Bcl-2 family protein function in yeast by exploring the morphological characteristics of *S. pombe* induced to die by Bax and its closely related homologue Bak, and asked whether Bax and Bak induce the production of ICE-/CED3-like protease activities in this unicellular organism. Finally, we established that Bax expression induces activation of apoptosis-associated ICE/CED-3-like protease activity in mammalian cells and compared this response to that seen in *S. pombe*.

MATERIALS AND METHODS

Yeast Strains and Media

The *S. pombe* strain SOP444 (*h*$^+$ *leu1–32 ura4-D18 his7–366 ade6-M210*) was used for all experiments. Cells were maintained in YEL/YES media before transformation and in EMM, lacking the selective amino acids, after transformation (Alfa *et al.*, 1993).

Cloning of Human Bak cDNA

The human *bak* cDNA was cloned by polymerase chain reaction (PCR) amplification from a HepG2 cDNA library using the primers 5′-ATTCCTGGAAACTGGGCTC-3′ and 5′-TGGAGTGCACCACTTGCTAAAG-3′. The resulting PCR product was digested with *Bam*HI and *Hin*dIII and cloned into the corresponding sites in Bluescript pSK-II (Stratagene, La Jolla, CA).

Plasmid Constructions

The vectors pREP3X (*leu2* marker) and pREP4X (*ura4* marker), containing the thiamine repressible *nmt* promoter, were used for conditional expression of cDNAs in *S. pombe* (Forsburg, 1993). All cDNAs were blunted and subcloned into the *Sma*I site of these vectors. The human *bcl-2* cDNA was excised from pRcCMV-Bcl-2 by digestion with *Xba*I and *Hin*dIII. The human Bax cDNA was obtained from pcDNA3-*hubax* by digestion with *Eco*RI. The *bcl-*X_L cDNA was taken from pSKIIBcl-X_L by *Eco*RI digestion. The human *bak* cDNA was excised from pSKII-Bak by digestion with *Bam*HI and *Hin*dIII. The p35 cDNA was excised from pPRM-3K-ORF (Sugimoto *et al.*, 1994) by digestion with *Bam*HI. The *bcl-2* (G145A) mutant was constructed by PCR amplification using the plasmid M1–3 (Yin *et al.*, 1994) as a template and the following primers: 5′-ATCAGTCTCGAGACTATGGCGCACGCTGGGAGA-3′ and 5′-ATCGATCTCGAGTCACTTGTGGCTCAGATAGGC-3′. The resulting PCR product was digested with *Xho*I and subcloned into pREP3X. The proper construction of all plasmids was confirmed by DNA sequencing.

Transformation and Induction of Protein Expression

Transformations were performed using the lithium acetate method (Moreno *et al.*, 1991). Cells were maintained in media (EMM lacking uracil and/or leucine) containing 5 μg/ml thiamine to prevent induction of the *nmt* promoter. Cells were then cultured either on plates or in liquid media in the presence or absence of thiamine. Cells in liquid culture were periodically diluted to sustain log phase growth.

Immunoblot Assays

Cells were harvested by centrifugation, washed once with phosphate-buffered saline, and resuspended in Laemmli buffer. The resulting lysates were kept at −70°C until used, boiled for 15 min, and briefly centrifuged, and the supernatants were run in 12% polyacrylamide gels. Samples were normalized for cell number, determined by optical density at OD_{600}. Immunoblotting was performed using an enhanced chemiluminescence method (Krajewski *et al.*, 1996b). Antibodies used for these studies included polyclonal rabbit antisera huBax 1701, huBak 1764, and huBcl-X 1695 and the anti-human Bcl-2 monoclonal antibody 4D7 (Krajewski *et al.*, 1994–1996a; Reed *et al.*, 1992).

Electron Microscopy (EM)

Cells were harvested at the indicated times by centrifugation, washed once with phosphate-buffered saline, and fixed for 30 min in 4 M phosphate buffer containing 3% glutaraldehyde. After two washing steps with 4 M phosphate buffer, cell pellets were embedded in Epon. Cells were postfixed and counterstained with 0.5% osmium tetroxide and 1% uranyl acetate, cut ultrathin, and placed on grids (Krajewski *et al.*, 1993). Sections were imaged using a Hitachi H-600 electron microscope.

Cysteine Protease Enzyme Assays

Lysates (50 μg total protein) were prepared from *S. pombe* Bax/p35, Bak/p35, and Rep/p35 transformants by glass bead disruption, adjusted to 150 μl total volume in buffer A [20 mM *n*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, pH 7.5, 1 mM EDTA, 5 mM dithiothreitol, 0.1% 3-([3-cholamidopropyl]dimethylammonio)-2-hydroxy-1-propanesulfonate, 10% sucrose], and added to a single well of a 96-well plate. Reactions were started by addition of 50 μl of fluorogenic tetrapeptide-aminomethylcoumarin (AMC) substrates to a final concentration of 10 μM. The following substrates were used: DEVD-AMC and YVAD-AMC. Fluorescent AMC product formation was measured at excitation 360 nm, emission 460 nm, using a cytofluor II fluorescent plate reader (Millipore, Bradford, MA). The change in fluorescence was measured over 1 h and expressed as the change in fluorescence per hour. 293 cell lysates were prepared in buffer A from 1.8×10^6 cells on 10-cm dishes 24 h after transfection using standard calcium phosphate methods with either 50 μg pCIneo (Promega, Madison, WI) alone, 50 μg total of pCIneo/pCIp35, pCIBax/pCIneo, or pCIBax/pCIp35 at equal molar ratios. Fifty micrograms of extract were used per assay point and determinations were made in duplicate.

p35 Inhibition Assays

Lysates (50 μg total protein) from *S. pombe* Bax/p35, Bak/p35, and Rep/p35 double transformants or pRep transformants as a control were prepared by glass bead disruption or, alternatively, 50 ng of recombinant 6Hisp35 protein were used. Yeast cell lysates or recombinant p35 were adjusted to a total volume of 100 μl in buffer A, added to a single well of a 96-well plate, and 1 U of recombinant purified active CPP32 protease (Nicholson *et al.*, 1995) was added in a volume of 75 μl. The substrate DEVD-AMC was then added in 25 μl of buffer A to a final concentration of 10 μM and release of the fluorogenic AMC product was monitored as described above. Inhibition of CPP32 activity by lysates or recombinant purified p35 protein was expressed as a percentage relative to the DEVD-AMC cleaving activity obtained when 1 U of CPP32 is combined with 75 μg of Rep3X control extract.

Mammalian Cell Viability Assay

293 cells (2×10^5/well) in 6-well plates were transfected by calcium phosphate precipitation with pCIneo/pCIBax/pCIp35 as appropriate in an equal molar ratio. One-fifth the amount of pRcCπV-β-galactosidase expression vector was cotransfected as a marker for transfected cells. Twenty-four hours after transfection, cells were fixed and *LacZ*-expressing cells were visualized using 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside as a substrate. Percentage of death was determined as the ratio of round blue cells to total (round + flat) blue cells × 100. Experiments were done in triplicate and are expressed as the mean value ± SD.

RESULTS

Expression of Bax or Bak Confers a Lethal Phenotype in S. pombe

cDNAs encoding the human Bax and Bak proteins were subcloned into the *S. pombe* vector pREP3X, which utilizes an *nmt* ("no message with thiamine") promoter for conditional expression of heterologous cDNAs. The presence of thiamine in the growth media represses expression of this promoter, whereas removal of thiamine induces the promoter with a lag time of ~12 h (Maundrell, 1990). *S. pombe* cells (strain SOP444) were transformed with the Bax and Bak expression plasmids or the parental pREP3X plasmid as a control. Transformants were initially plated on media containing thiamine, thus repressing the *nmt* promoter, and then single colonies were isolated, streaked onto plates containing or lacking thiamine, and assayed for their ability to grow. As shown in Figure 1A, yeast calls carrying the Bax expression plasmid were markedly impaired in their ability to grow on plates lacking thiamine compared with cells containing the control plasmid. In contrast, transformants containing the Bax and control plasmids grew equally well on thiamine-supplemented medium. Similar results were obtained for Bak (our unpublished results).

To determine whether Bax and Bak induce cell death as opposed to a reversible growth arrest,yeast cells that had been transformed with the Bax, Bak, or control plasmids were cultured in liquid media without thiamine to induce the *nmt* promoter. Aliquots of these cultures were then removed at various times and plated onto solid media containing thiamine. If the growth inhibition mediated by Bax and Bak was reversible, then plating the cells on thiamine-containing medium would be expected to rescue the cells and result in colony-forming units at frequencies comparable to cells carrying the control plasmid. Conversely, if Bax and Bak kill *S. pombe*, then the cells should not be rescuable on thiamine-supplemented medium.

As shown in Figure 2,A and B, culturing cells that contained Bax or Bak expression plasmids in thiamine-deficient medium (to induce the *nmt* promoter) resulted in a time-dependent decline in the numbers of viable clonigenic cells that could be subsequently recovered on plates containing thiamine, with >75% inhibition of viable colony formation within ~10 h and nearly complete suppression of colony formation occurring within ~14 h (data normalized relative to efficiency of rescue of cells containing the pREP parental plasmid as a control). Using trypan blue staining, we observed the appearance of dying cells around 10–14 h (our unpublished results), consistent with the plating results. These data are consistent with a lethal effect of Bax and Bak on *S. pombe*.

Bcl-2 and Bcl-XL Rescue S. pombe from Lethal Effects of Bax and Bak

Bcl-2 and Bcl-X_L are anti-apoptotic members of the Bcl-2 protein family that have been reported to suppress the cell death-promoting effects of Bax and Bak in mammalian cells (Oltvai *et al.*, 1993; Chittenden *et al.*, 1995a,b; Farrow *et al.*, 1995). Bcl-X_L can heterodimerize with both Bax and Bak, whereas Bcl-2 interacts efficiently with Bax but more poorly with Bak (Farrow *et al.*, 1995). Certain loss-of-function mutants of Bcl-2, such as Bcl-2 (G145A), fail to heterodimerize with Bax and also do not suppress apoptosis (Yin *et al.*, 1994). To determine whether the expression of Bcl-2 or Bcl-X_L can rescue fission yeast from Bax- or Bak-induced cell death, *S. pombe* cells containing Bax or Bak expression plasmids were transformed with expression plasmids encoding wild-type Bcl-2, mutant Bcl-2(G145A), or Bcl-X_L under the control of the *nmt* promoter in pREP4X which carries a URA4 selectable marker as opposed to the LEU2 marker in pREP3X plasmid from which Bax and Bak were expressed.

After initial plating on thiamine-containing medium to repress the *nmt* promoter and thus prevent expression of Bax and Bak, single colonies of these transformants were streaked onto plates that contained or lacked thiamine. Both Bcl-2 and Bcl-X_L restored growth to Bax- and Bak-expressing cells when plated on thiamine-deficient medium, as illustrated by the representative experiment shown in Figure 1B. In contrast, Bax- and Bak-expressing cells that contained the plasmid encoding the mutant Bcl-2 (G145A) protein failed to grow when streaked on thiamine-deficient medium. Comparable growth was observed for all transformants when streaked onto plates containing thiamine-supplemented medium, demonstrating the specificity of these results (Figure 1B). Bcl-X_L consistently afforded more protection than Bcl-2 in these assays, not unlike some results involving mammalian cells (Gottschalk *et al.*, 1994). Similar results were obtained with ~10 other independent clones of each transformation.

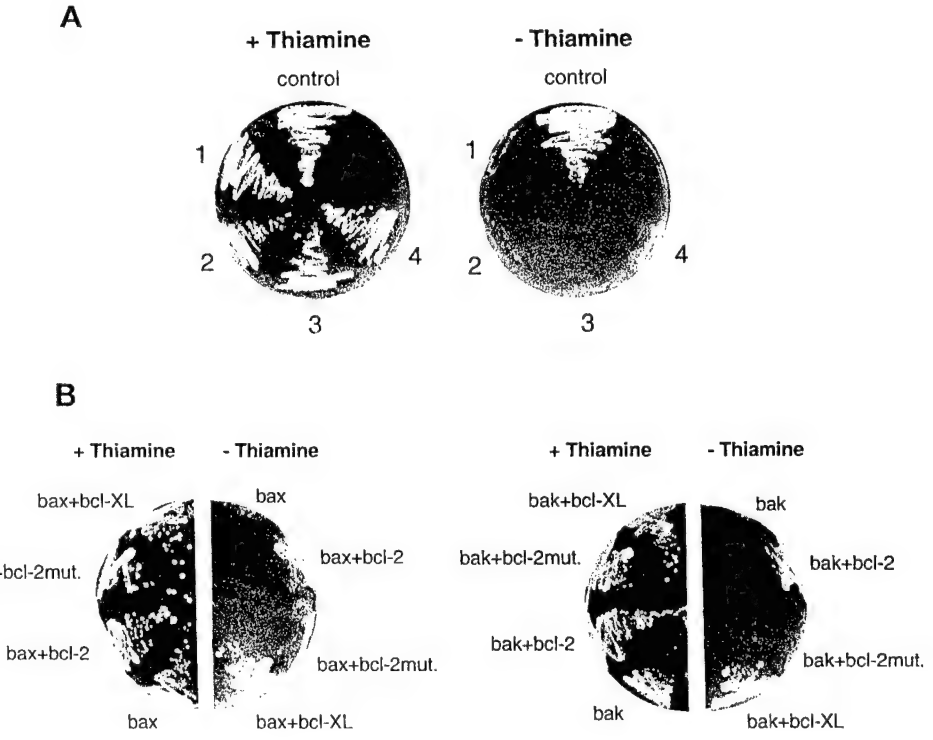

Figure 1. Bax/Bak-induced cell death in *S. pombe* and rescue by Bcl-2 and Bcl-X_L. In A, *S. pombe* cells were transformed with a Bax expression plasmid. Four independent clones were tested on selective media with and without thiamine (1–4). In the absence of thiamine Bax is expressed. Controls were transformed with the parent plasmid lacking a *bax* cDNA insert. In B, individual colonies of *S. pombe* transformants containing pREP3X-Bax or pREP3X-Bax with either pREP4X, pREP4X-Bcl-2, pREP4X-Bcl-2 mutant (G145A), or REP4X-Bcl-X_L plasmids were streaked onto plates containing or lacking thiamine and growth was monitored 4 d later.

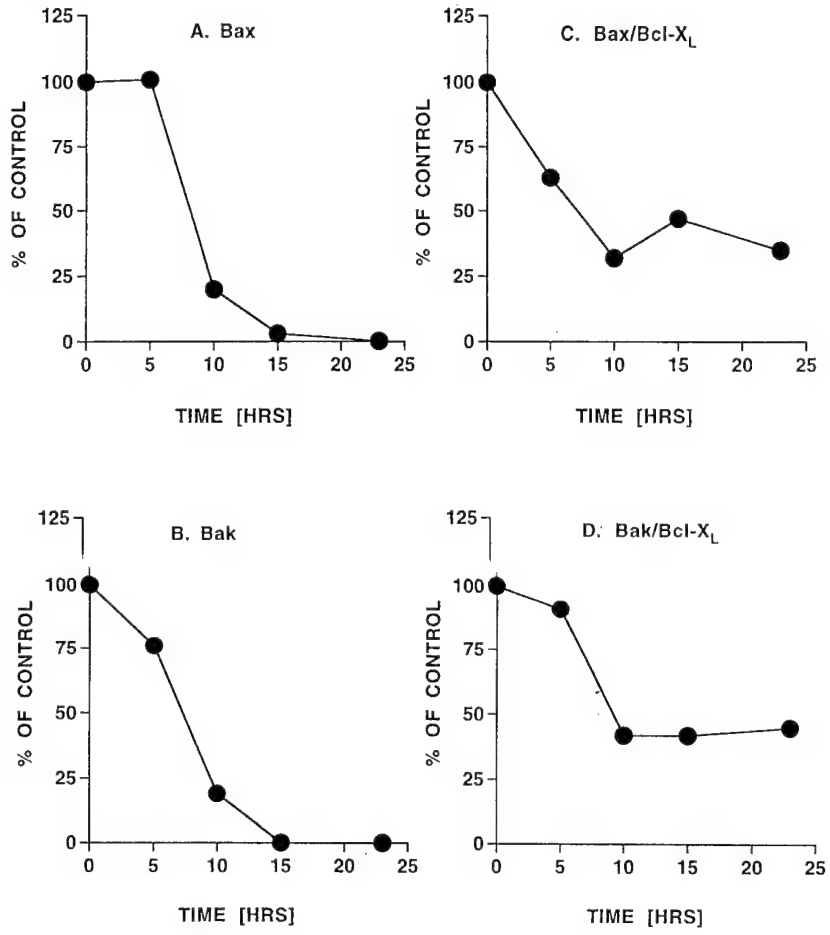

Figure 2. Bax- and Bak-mediated growth inhibition is irreversible. Cells were transformed with pREP3X and pREP4X plasmids either lacking a cDNA insert (parental) or containing *bax*, *bak*, or *bcl-X$_L$* cDNAs and grown to mid-log phase in thiamine-containing medium, and then washed and diluted 1:10 into fresh medium lacking thiamine to induce the *nmt* promoter. At various times thereafter, cells were plated onto thiamine-containing solid medium, and the relative numbers of colonies were determined based on comparisons with cells cotransformed with the pREP3X and pREP4X parental plasmids. (A) Bax; (B) Bak; (C) Bax and Bcl-X$_L$; (D) Bak and Bcl-X$_L$.

The ability of Bcl-X$_L$ and Bcl-2 to rescue Bax- and Bak-expressing *S. pombe* cells was also observed in experiments where cells were transiently cultured in thiamine-deficient medium to induce the *nmt* promoter in the pREP3X and pREP4X plasmids that contained *bax*, *bak*, *bcl-2*, and *bcl-X$_L$* cDNAs, and then at various times thereafter were replated onto thiamine-containing medium to shutoff expression of Bax and Bak. As shown in Figure 2, 25–50% of the cells that had been cotransformed with the pREP4X-Bcl-X$_L$ plasmid and either the pREP3X-Bax (Figure 2C) or the pREP3X-Bak (Figure 2D) plasmid were rescuable when plated on thiamine-containing plates after a period of growth in thiamine-deficient medium. Similar results were obtained with Bcl-2, but the rescue was less effective compared with Bcl-X$_L$ (our unpublished results). In contrast, the cells transformed with the pREP4X parental plasmid (lacking a *bcl-X$_L$* or *bcl-2* cDNA) and either pREP3X-Bax or pREP3X-Bak were all unrecoverable within 24 h of growth in thiamine-deficient medium, as mentioned above (Figure 2, A and B). Since coexpression of Bcl-X$_L$ allowed cells to survive a transient exposure to Bax or Bak, resulting in increased numbers of viable clonigenic cells, we conclude that Bcl-X$_L$ is capable of abrogating the lethal effects of Bax and Bak in *S. pombe*.

Finally, the effects of Bcl-2 and Bcl-X$_L$ on Bax- and Bak-expressing *S. pombe* cells were examined by growing cells in thiamine-deficient liquid culture medium and spectrophotometrically assessing the cell culture densities over a ~2-day period by measuring the absorbance at 600 nm. As shown in Figure 3, when *S. pombe* cells containing the pREP3X-Bax or pREP3X-Bak plasmids in combination with the pREP4X control plasmid lacking a *bcl-2* or *bcl-X$_L$* cDNA were switched from thiamine-containing medium to thiamine-free medium, growth as defined by $OD_{600\ nm}$ began to cease by ~ 24 h. In contrast, the cell densities in cultures of *S. pombe* cells that contained the pREP4X-Bcl-X$_L$ or pREP4X-Bcl-2 expression plasmids along with either pREP3X-Bax or pREP3X-Bak continued to increase at 24 h, with faster rates of growth seen in the Bcl-X$_L$- than the Bcl-2-expressing cells (Figure 3, A and B). Cells that coexpressed the Bcl-2 (G145A) mutant protein with either Bax or Bak, on the other hand, ceased growing after ~24 h, thus verifying the speci-

ficity of the results. Growth of all transformants was comparable in thiamine-supplemented medium (our unpublished results), consistent with the thiamine-mediated repression of the *nmt* promoter.

As an additional control, we examined the effects of Bcl-2 and Bcl-X_L on the growth of *S. pombe* in the absence of Bax or Bak. Cells transformed with the pREP4X-Bcl-2 plasmid and pREP3X, the same parental plasmid from which Bax and Bak were expressed but without the *bax* or *bak* cDNAs, grew at rates comparable to control transformants containing only the pREP4X and pREP3X parental plasmids (Figure 3C). Thus, Bcl-2 by itself did not have an effect on growth. In contrast, yeast cells transformed with pREP4X-Bcl-X_L and pREP3X grew somewhat faster than control transformants containing the pREP4X and pREP3X parental plasmids in some experiments (e.g., Figure 3), but grew at rates essentially identical to control transformants in others. Taken together, these data in Figure 3 support the contention that Bcl-2 and Bcl-X_L specifically suppress the lethal effects of Bax and Bak in *S. pombe*, whereas the Bcl-2 (G145A) mutant does not.

Time Courses of Bak and Bax Protein Accumulation Correlate with Kinetics of Growth Inhibition in S. pombe

The time courses of Bax, Bak, Bcl-X_L, and Bcl-2 protein accumulation were evaluated by immunoblotting of the various *S. pombe* transformants after inducing cells in thiamine-deficient medium. These experiments were performed in parallel with the growth studies described above in Figure 3, thus allowing direct comparisons of expression of these Bcl-2 family proteins with function. As shown in Figure 4, production of the Bax and Bak proteins was either undetectable or just barely detectable by immunoblotting at the earliest time examined (14 h) but became maximal at 24–36 h after seeding cells into thiamine-deficient medium. When compared with the data above in Figure 3, these immunoblotting data therefore suggest that accumulation of the Bax and Bak proteins coincides roughly with the onset of growth inhibition. The observation that 10–15 h of Bax or Bak induction renders cells nonviable (Figure 2) suggests that these proteins can have effects on *S. pombe* cells at modest expression levels.

The time courses of induction of Bcl-2 and Bcl-X_L were similar to those of Bax and Bak, as might be expected given that the same *nmt* promoter was used for driving expression of these anti-apoptotic proteins in *S. pombe*. Note that the wild-type and mutant Bcl-2 (G145A) proteins were produced at comparable levels in yeast, thus excluding insufficient production of the mutant Bcl-2 (G145A) protein as an explanation for its failure to rescue cells from Bax- and Bak-induced death. The levels of Bax and Bak produced in yeast containing the Bcl-X_L or Bcl-2 expression plasmids

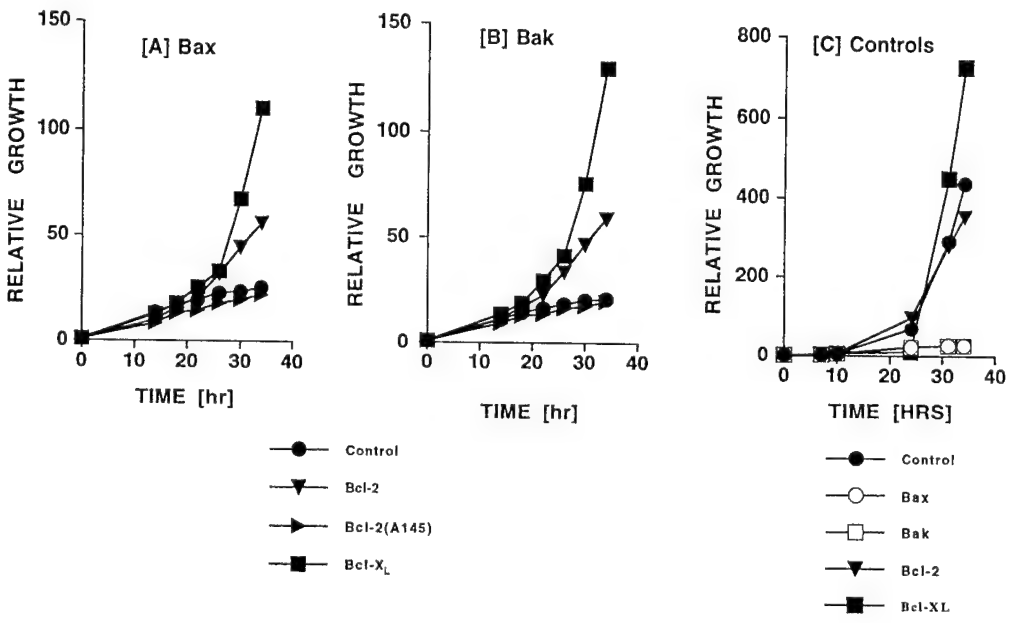

Figure 3. Time course of effects of Bax and Bak on growth of *S. pombe*. Cells transformed with pREP3X plasmids containing *bax* or *bak* cDNAs with either pREP4X, pREP4X-Bcl-2, pREP4X-Bcl-2 mutant (G145A), or pREP4X-Bcl-X_L plasmids were grown to mid-log phase in thiamine-containing medium, and then washed and diluted 1:10 into fresh medium lacking thiamine to induce the *nmt* promoter. At various times thereafter, relative cell growth was estimated by $OD_{600\ nm}$. Cells were maintained in mid-log phase growth throughout the experiment by dilution in fresh thiamine-deficient medium prior to the $OD_{600\ nm}$ reaching 0.7. Data represent calculated theoretical total ODs. C (control) represents cells cotransformed with the pREP3X and pREP4X control plasmids. In A, all transformants contained the pREP3X-Bax plasmid along with various pREP4X plasmids as indicated. In B, all transformants contained the pREP3X-Bak plasmid along with other plasmids. In C, cells cotransformed with pREP3X parental plasmid and either pREP4X parental plasmid (C, ●), pREP4X-Bcl-2 (○), or pREP4X-Bcl-X_L (■). Alternatively, cells were transformed with the pREP4X parental plasmid and either pREP3X-Bax (□) or pREP3X-Bak or pREP3X-Bax (□).

were typically comparable to or even greater than those seen in cells that expressed Bax or Bak in the absence of these anti-apoptotic proteins, thus discounting lower levels of Bax and Bak protein as the explanation for the rescue of *S. pombe* by Bcl-X_L and Bcl-2 (our unpublished results). Moreover, the maximum relative levels of Bax and Bak reached in fission yeast cells were comparable to those seen in several mammalian tissues when samples were normalized for total protein content (Krajewski *et al.*, 1996a, and our unpublished results). Thus, it cannot be argued that supraphysiological levels of these cell death-promoting proteins were obtained in *S. pombe*. Analysis of control transfected *S. pombe* cells that contained the same pREP 3X and pREP 4X plasmids without *bax*, *bak*, *bcl-*X_L, or *bcl-2* cDNA inserts ("C") revealed no proteins that could be detected by antibodies directed against the human proteins, thus confirming the specificity of the immunoblotting results (Figure 4).

Morphological Analysis of Bax- and Bak-expressing S. pombe *by EM*

The distinct morphological features that characterize apoptosis in animal cells are best demonstrated by transmission EM (reviewed in Wyllie *et al.*, 1980). We therefore analyzed by EM the morphology of *S. pombe* cells undergoing cell death as a result of expressing Bax or Bak. As shown in Figure 5, striking differences were seen in the morphology of control cells that carried plasmids lacking cDNA inserts (A) and the Bax-expressing *S. pombe* cells (B). Bax-expressing cells uniformly developed massive vacuolization of the cytosol. The cytosol also became electron dense ("cytosolic condensation"), similar to previous descriptions of programmed cell death in plant and animal cells. However, the size distribution of control and dying Bax-expressing cells was approximately the same, and thus Bax expression did not induce the cell shrinkage typical of mammalian cell apoptosis. Also, unlike apoptosis in animal cells, plasma membrane blebbing was not observed, but at higher resolution (our unpublished results) invaginations of the plasma membrane were commonly present beneath the cell wall in Bax- and Bak-expressing cells. Foci of chromatin condensation were present in the nucleus of Bax-expressing cells but not in the control cells. No evidence of nuclear fragmentation or of chromatin margination against the nuclear envelope was obtained by EM

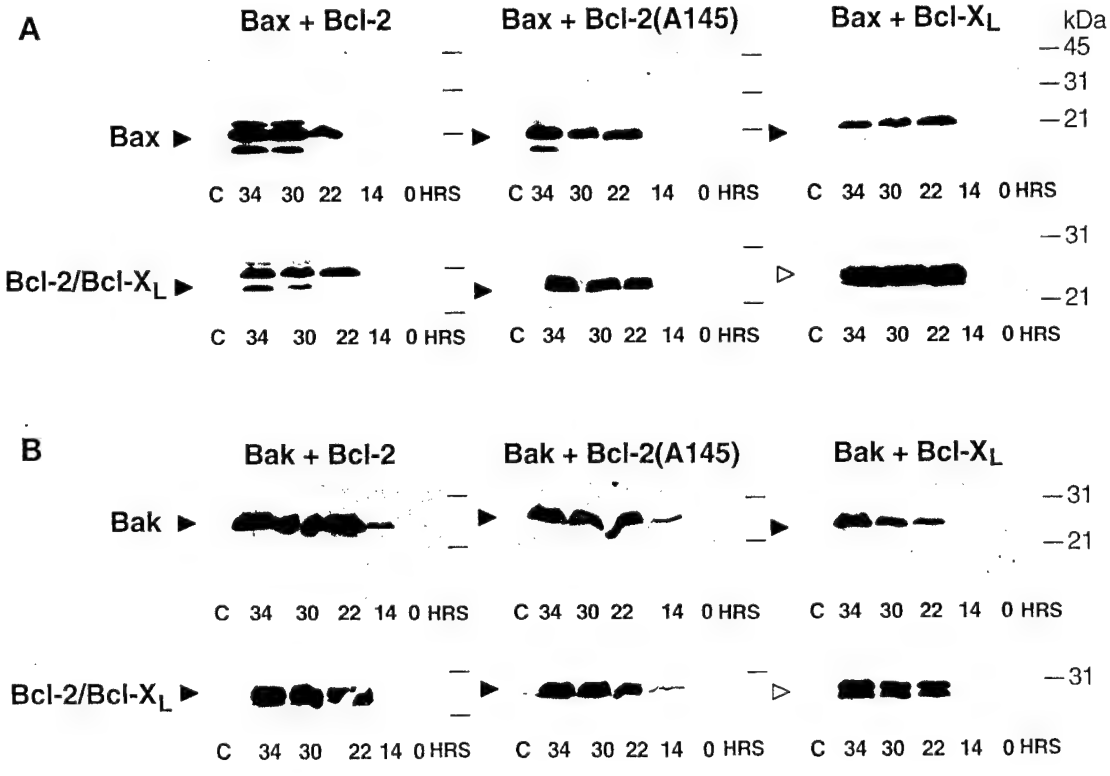

Figure 4. Immunoblot analysis of Bcl-2 family proteins in *S. pombe*. Cells that had been cotransformed with pREP3X plasmids encoding either Bax or Bak and REP4X plasmids encoding Bcl-2, Bcl-2 (G145A), or Bcl-X_L were grown as described in Figure 3, and aliquots of the cells were removed, lysed, and their proteins analyzed by SDS-PAGE/immunoblotting using antisera specific for the human Bax, Bak, Bcl-2, and Bcl-X proteins (Krajewski *et al.*, 1996b). Detection was accomplished with an enhanced chemiluminescence method using a horseradish peroxidase-conjugated secondary goat anti-rabbit antibody as described (Krajewski *et al.*, 1996b). C represents control cells cotransformed with pREP3X and pREP4X parental plasmids. In the rows labeled as Bcl-2/Bcl-X_L, the closed arrows indicate the positions of the Bcl-2 protein, whereas the open arrows indicate the position of the Bcl-X_L protein. The Bcl-X_L protein routinely migrates as a doublet in SDS-PAGE (Krajewski *et al.*, 1994). The smaller band seen in some cases for Bcl-2 probably represents a partial degradation product.

analysis. Similar results were obtained for Bak-expressing *S. pombe* cells (our unpublished results). Despite the presence of focal chromatin condensation in Bax- and Bak-expressing *S. pombe* cells, we were unable to detect the presence of fragmented DNA having the oligonucleosomal pattern typical of apoptotic cells by agarose gel electrophoresis.

To explore whether these morphological changes were specifically associated with the cell death process induced by Bax and Bak versus merely a characteristic of producing Bax or Bak protein in this fission yeast, EM analysis was performed for *S. pombe* cells coexpressing Bcl-X_L with either Bax or Bak. Immunoblot analysis confirmed that the relative levels of the Bax and Bak proteins produced in these Bcl-X_L-expressing cells were as high as those seen in cells expressing Bax or Bak alone without Bcl-X_L (our unpublished results). In fact, the levels of Bax and Bak tended to be approximately twofold to threefold higher in the Bcl-X_L-expressing cells (our unpublished results), possibly because these cells were able to tolerate higher levels of these proapoptotic proteins. EM analysis of these cells revealed that about one-half to three-quarters of the Bcl-X_L-expressing cells retained the morphological features of control cells, whereas the others developed the cytosolic condensation, vacuolization, and focal chromatin condensation that was seen in essentially all of the cells that expressed Bax or Bax alone in the absence of Bcl-X_L (Figure 5C). These morphological data are in general accord with the clonogenic assay (Figure 2) which also demonstrates a partial rescue from Bax/Bak-induced death. The incomplete rescue in these assays is possibly due to clonal differences in the copy number of the episomal plasmid from which Bcl-X_L was expressed.

These morphological features of dying *S. pombe* cells were not unique to Bax and Bak, since overexpression of the protein tyrosine phosphatase pyp1 produced many of the same changes but with a delay of ~1 d relative to Bax and Bak. The pyp1 phosphatase induces a G_2-M arrest followed by cell death (Ottilie *et al.*, 1992). EM analysis of pyp1-expressing cells demonstrated elongation of the cells consistent with a G_2-M arrest (our unpublished results), followed ~1 d later by the massive vacuolization and other changes seen in Bax- and Bak-expressing *S. pombe* cells. Multifocal chromatin condensation, however, was less evident in these pyp1-expressing cells compared with Bax and Bak (our unpublished results). Similar morphological features have been described recently for *S. cerevisiae* overexpressing histone H1 (Miloshev *et al.*, 1994), further suggesting that the morphology produced as a result of ectopic expression of Bax and Bak in *S. pombe* is not unique to these mammalian proapoptotic proteins.

Absence of Detectable ICE/CED-3-like Protease Activity in S. pombe Cells Undergoing Bax- and Bak-mediated Cell Death

The induction of apoptosis in mammalian cells is typically accompanied by the activation of proteases of the ICE/CED-3 family (reviewed in Martin and Green, 1995). These proteases cleave their substrates specifically after aspartic acid. To address the question of whether *S. pombe* cells undergoing cell death due to the expression of *bax* contain similar protease activities, lysates were prepared from cells after inducing Bax expression from the *nmt* promoter in pREP3X as well as from control cells containing the same plasmid without Bax. In vitro assays for ICE/CED-3 protease activity were then performed using two different fluorogenic peptide substrates that are known to be effective for one or more ICE/CED-3 family proteases: YVAD-AMC and DEVD-AMC (Nicholson *et al.*, 1995). Neither of these substrates was cleaved by *S. pombe* lysates expressing Bax (Figure 6A). In contrast, Bax

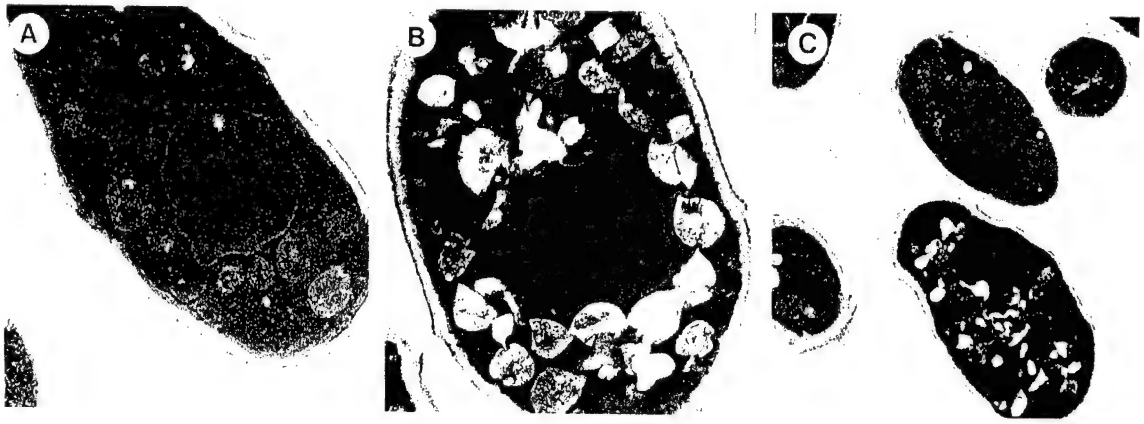

Figure 5. EM analysis of Bax-expressing *S. pombe*. Cells were transformed with either pREP3X (A), pREP3X-Bax (B), or pREP3X-Bax plus pREP4X-Bcl-X_L (C) and then grown for 1 d in thiamine-deficient medium prior to fixing cells and performing EM analysis.

overexpression in mammalian 293 cells leads to an increase in DEVD-AMC cleaving activity (Figure 6B).

p35 Does not Rescue Bax-induced Cell Death in S. pombe

To further explore the possibility that ICE/CED-3-like proteases are involved in the Bax/Bak-mediated cell death process in *S. pombe*, we constructed an expression plasmid containing a cDNA for the baculovirus *p35* gene under the control of the *nmt* promoter. The p35 protein binds to and inhibits the enzymatic activities of all known ICE/CED-3 family proteases (Rabizadeh *et al.*, 1993; Hay *et al.*, 1994; Sugimoto *et al.*, 1994; Bump *et al.*, 1995; Xue and Horvitz, 1995; Bertin *et al.*, 1996). The p35 protein has also been shown to block apoptosis and programmed cell death in a wide range of animal species or cells derived from them, including nematodes, flies, ants, mice, rats, and humans (Clem and Miller, 1994; Pronk *et al.*, 1996; White *et al.*, 1996). Yeast containing the pREP3X-Bax, pREP3X-Bak, or pREP3X plasmids were transformed with pREP4X-p35 or the same vector without a cDNA insert as a control. Cells were then grown to mid-log phase in thiamine-deficient medium to induce the expression of p35 and Bax and Bak. Cell lysates were prepared and tested for their ability to inhibit the activity of the ICE/CED-3-family protease CPP32 using an in vitro protease assay. These experiments demonstrated that an inhibitory activity consistent with p35 was specifically produced in the cells transformed with the p35 but not in cells transformed with the parental plasmid pRep (lysates normalized for total protein content). As an additional control, recombinant purified p35 protein (2.5 ng) was added to this in vitro protease assay, resulting in ~99% inhibition of CPP32 activity (Figure 6C).

Despite the production of biologically active p35 in yeast, no suppression of the lethal phenotype conferred by Bax and Bak was observed in cells that coexpressed p35 with these proapoptotic proteins (our unpublished results). Similarily, Bax- and Bak-induced lethality were also not impaired in yeast in which p35 expression was driven by a constitutive ADH promoter (our unpublished results). In contrast, in 293 cells both the Bax-induced DEVD-AMC cleaving activity (Figure 6B) and Bax-induced cell death are inhibited by overexpression of p35 (Figure 6D). Taken together, these results suggest that *S. pombe* lack protease activities similar to the ICE/CED-3 family proteases that become activated during apoptosis in animal cells. Furthermore, such proteases are evidently not the effectors of cell death induced by Bax and Bak in fission yeast.

DISCUSSION

The purpose of this study was to investigate the effects of Bax and Bak expression in the fission yeast *S. pombe* and to compare it to Bax-induced cell death in mammalian cells. Several observations demonstrate similarities between the effects of these proteins in fission yeast and mammalian cells. First, Bax and Bak expression resulted in cell death in yeast. Moreover, the effects of Bax and Bak were not related simply to an arrest of cell growth, but rather were due to cell death, as determined by clonigenic assays. Second, the lethal effect of Bax and Bak was specifically abrogated by coexpressing the anti-apoptotic proteins Bcl-X_L and Bcl-2, but not a mutant of Bcl-2 that fails to suppress the proapoptotic effects of Bax in animal cells (Yin *et al.*, 1994). These observations therefore are similar to reports involving mammalian cells, where the effects of these Bcl-2 family proteins on cell survival and death have been studied previously. Other observations, however, demonstrated differences between yeast and mammalian cells with regard to the cell death phenotype conferred by Bax and Bak. First, the morphology of *S. pombe* cells undergoing Bax/Bak-induced cell death involved cytoplasmic vacuolization, cytosolic condensation, and multifocal nuclear condensation, but not the cell shrinkage, nuclear fragmentation, and chromatin margination characteristic of apoptosis. Second, internucleosomal DNA fragmentation was not observed in this fission yeast, but is often found in mammalian cells undergoing apoptosis. Third, no ICE/CED-3-like protease activities were detected in yeast induced to express Bax or Bak. Fourth, the protease inhibitor p35 failed to prevent Bax/Bak-induced cell death in *S. pombe*.

The cell death process mediated by Bax and Bak in fission yeast involved morphological changes that were not consistent with apoptosis as defined in mammalian cells, but instead was more reminiscent of programmed cell death in plants and protists. In particular, the striking cytoplasmic vacuolization, cytosolic condensation, and multifocal nuclear condensation seen in *S. pombe* when induced to die by Bax and Bak were highly similar to the programmed cell death reported previously for the slime mold *Dictyostelium* (Cornillon *et al.*, 1994). Like the death of stalk cells seen in the multicellular structures formed by *Dictyostelium* during times of nutritional insufficiency, the induction of cell death by Bax and Bak in *S. pombe* did not involve DNA fragmentation in the oligonucleosomal pattern that is typical of apoptosis in many types of animal cells. Since pulse-field gel electrophoretic analysis of yeast chromosomal DNA was not performed, we cannot exclude the possibility that DNA fragmentation into higher molecular weight fragments occurred, as has been reported for some types of mam-

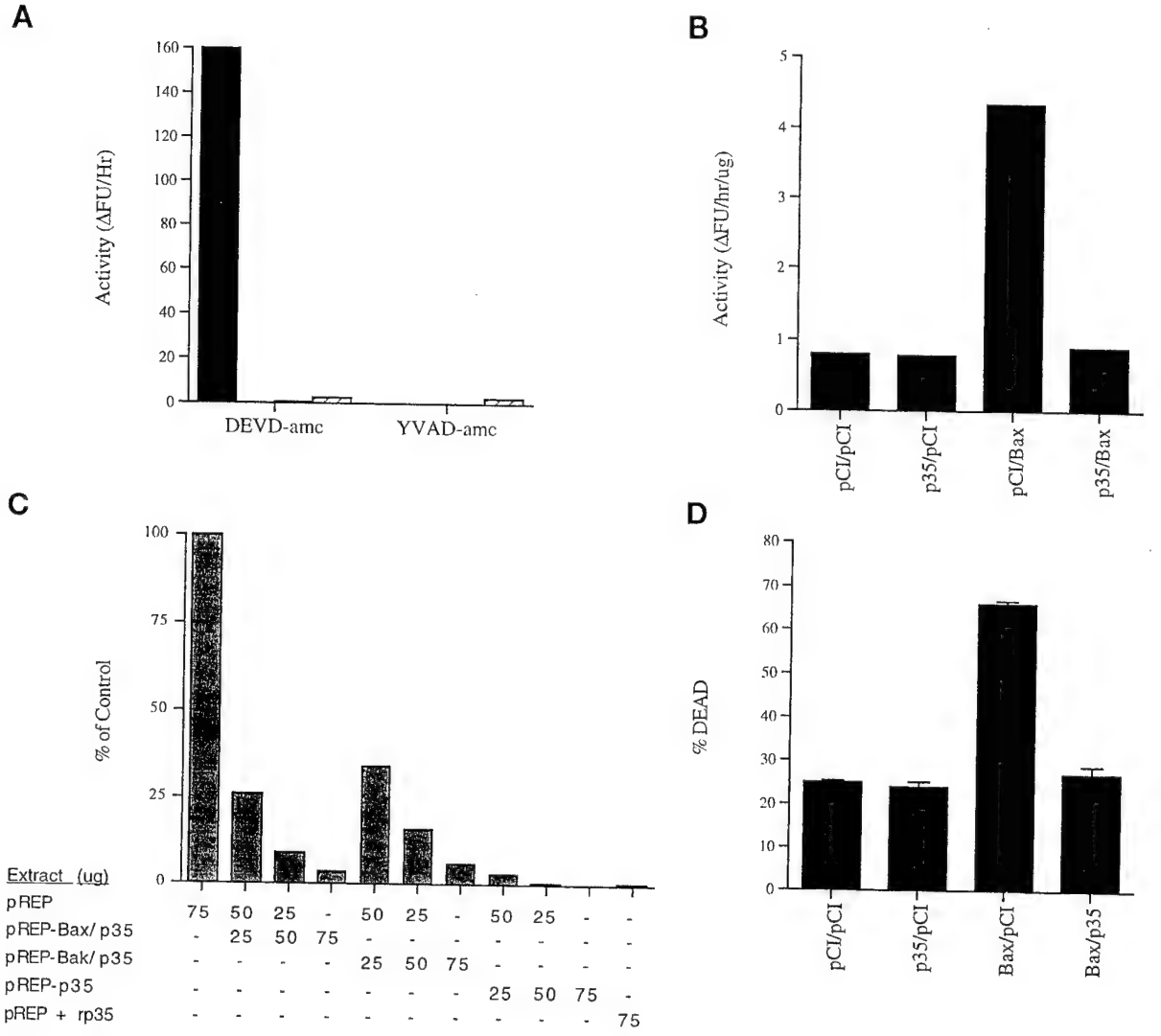

Figure 6. Lack of ICE/CED-3-like protease activity in Bax-transformed *S. pombe* cells. In A, lysates were prepared from *S. pombe* cells cotransformed with pREP4X and either the pREP3X parental plasmid (hatched bar; no activity) or pREP3X-Bax (stippled bar) at 12 h after diluting them into thiamine-deficient medium. The relative amount of release of fluorogenic product (AMC) from the peptide substrates DEVD-AMC and YVAD-AMC in 1 h is shown in arbitrary fluorescence units (ΔFU). As a control, 1 U of purified recombinant CPP32 (solid bar) is shown using DEVD-AMC as a substrate. (This protease cleaves the peptide substrate DEVD-AMC (Nicholson *et al.*, 1995.) In B, 293 lysates were prepared 21 h after transfection with 50 μg of pCIneo or 25 μg each of pCIneo/p35, Bax/pCIneo, or Bax/p35. The relative amount of release of fluorogenic product (AMC) from peptide substrate (DEVD-AMC) (ΔFU) in 1 h is shown in arbitrary fluorescence units. In C, *S. pombe* cells were transformed with either pREP3X-Bax, pREP3X-Bak, or pREP3X parental plasmid, with or without pREP4X-p35 as indicated. Cell lysates were prepared and tested for their ability to inhibit CPP32-mediated cleavage of DEVD-AMC. Specified amounts of each extract were mixed and their ability to inhibit rCPP32 activity was assayed. Results are expressed as percentage of control which corresponds to the activity obtained when rCPP32 is combined with extract from control vector-transformed cells (pREP). The degree of inhibition obtained by addition of 2.5 ng of affinity-purified 6His recombinant p35 (rp35) is included for comparison. In D, 293 cells were transfected as in B except 1 μg of pRcCMV-LacZ *LacZ* expression vector was included with each transfection. Twenty-four hours after transfection, cells were fixed and the presence of functional *LacZ* was detected with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside. Percentage of dead cells was determined as the ratio of round (dead) blue cells to flat (live) blue cells.

malian cells when undergoing apoptosis (Oberhammer *et al.*, 1993; Gromova *et al.*, 1995; Lagarkova *et al.*, 1995). Such DNA fragmentation however was not detected in *Dictyostelium*, suggesting that programmed cell death can occur in at least some types of protist cells in the absence of nonrandom DNA degradation.

Of relevance to this issue, genetic analyses of the nematode *C. elegans*, as well as investigations of some mutant mammalian cell lines, have suggested that genomic digestion is not a necessary requirement for programmed and apoptotic cell death in animal cells (reviewed in Ellis, 1991; Ucker, 1991).

The morphological features of yeast dying as a result of Bax or Bak expression were not unique to these proapoptotic Bcl-2 family proteins. For example, overexpression of the protein tyrosine phosphatase gene *pyp1*$^+$ in fission yeast resulted in a similar phenotype, but with a longer delay compared with Bax and Bak. Furthermore, cell death induced in the budding yeast *S. cerevisiae* by overexpression of histone H1 produces a very similar morphology (Miloshev *et al.*, 1994). Thus, the cell death processes induced by a variety of stressors, including Bax and Bak, may ultimately culminate in a similar morphological phenotype in yeast.

The cell death induced by Bax and Bak in *S. pombe* differed from Bax-induced cell death in mammalian cells in that no ICE/CED-3-like protease activities were detected and the baculovirus p35 protein could not protect fission yeast from the lethal effects of Bak and Bax. In the nematode, *C. elegans*, loss-of-function mutations in *ced-3*, which encodes a cysteine protease with homology to ICE, results in a failure of all 131 programmed cell deaths that normally occur in this animal during development (Yuan *et al.*, 1993). Conversely, loss-of-function mutations in the *ced-9* gene, the worm homologue of *bcl-2*, cause massive developmental cell death, which can be blocked by the baculovirus p35 protein (Hengartner *et al.*, 1992; Sugimoto *et al.*, 1994). Ectopic expression of the p35 protein in the eyes of transgenic flies also protects from cell death induced by overexpression of the cell death genes *reaper* and *hid* (Pronk *et al.*, 1996; White *et al.*, 1996). In human cell lines, apoptosis induced via the cytokine receptors Fas/APO-1 (CD95) and tumor necrosis factor receptor type I can also be prevented by p35 (Beidler *et al.*, 1995). The p35 protein plays an important role in the normal life cycle of baculoviruses, which depend at least in part on p35 for preventing cell death and thus allowing viral replication to occur in infected insect cells (Clem and Miller, 1994). The fact that p35 can rescue mammalian 293 cells from apoptosis induced by Bax expression but cannot suppress Bax- and Bak-induced cell death in *S. pombe* suggests that the downstream events leading to cell death in fission yeast when expressing Bax or Bak are distinct from those occurring after Bax overexpression in animal cells.

These observations raise the possibility that the ICE/CED-3 family cysteine proteases might have arisen at a relatively late point in the evolution of cell death suicide mechanisms, after the division of the plant and animal kingdoms. It should be noted, however, that other types of cell death-associated aspases may be present in yeast which are both undetectable by the particular protease assays used here and are uninhibitable by p35. For example, cytolytic T-cells in humans and rodents produce a serine protease, granzyme B, with specificity for aspartic acid in the P1 position of substrates which is a potent inducer of apoptosis but which is not inhibited by p35 (Bump *et al.*, 1995; Quan *et al.*, 1995; Tewari *et al.*, 1995). Also, it remains unknown at present whether all programmed or apoptotic cell deaths in animal species require the actions of ICE/CED-3-like aspases.

The findings that 1) Bax can induce cell death in both fission and budding yeast and that 2) Bcl-X_L and Bcl-2 can rescue yeast from the lethal actions of this proapoptotic protein raise the possibility that at least some portions of the cell death mechanism controlled by Bcl-2 family proteins may be evolutionarily conserved even beyond animal species to simple unicellular eukaryotes and perhaps plants. The question then is to what extent the biochemical processes regulated by Bcl-2 family proteins in yeast resemble those that are relevant to cell death control in animal cells. Similar to reports on mammalian cells, we observed that a mutant version of Bcl-2 (G145A) which fails to heterodimerize with Bax was incapable of suppressing Bax- or Bak-induced cell death in the fission yeast *S. pombe*. Also, a variety of loss-of-function mutants of Bcl-2 that either do or do not retain Bax-binding activity were previously shown to be inactive at suppressing Bax-mediated lethality in the budding yeast *S. cerevisiae* (Hanada *et al.*, 1995). Moreover, as shown here, the Bak protein also functioned as a cell death inducer in *S. pombe*, despite the fact that it shares only 19% amino acid sequence homology with Bax (Kiefer *et al.*, 1995). In this regard, the recently reported three-dimensional structure of the Bcl-X_L protein has suggested that Bcl-2 family proteins may be pore-forming molecules with similarity to certain bacterial toxins such as diphtheria toxin and bacterial colicins (Muchmore *et al.*, 1996). Thus, the cell death mechanism induced in *S. pombe* and *S. cerevisiae* by Bax and Bak potentially may be a reflection of an intrinsic activity of these proteins that requires no further cooperating proteins from yeast.

Although these observations could be taken as evidence that Bax/Bak-induced cell death in fission and budding yeast has close parallels with mammalian systems, it is also possible that Bax and Bak trigger cell death through mechanisms that are biochemically dissimilar in yeast and animal cells. In this regard, we have been unable to identify homologues of Bcl-2 and Bax in yeast by low-stringency hybridization and two-hybrid screening of genomic libraries, raising the possibility that yeasts do not normally rely on such proteins for regulating cell survival and death. These caveats notwithstanding, the data reported here and elsewhere (Sato *et al.*, 1994; Hanada *et al.*, 1995; Greenhalf *et al.*, 1996) showing biological effects of Bcl-2 family proteins in yeast, when taken together with the recent x-ray crystallographic and NMR structural data for Bcl-X_L, suggest that yeast may provide a convenient background in which to perform certain types of structure-function analyses of Bcl-2 family proteins.

Moreover, these simple organisms may provide important opportunities for probing the evolutionary origins of programmed cell death mechanisms.

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